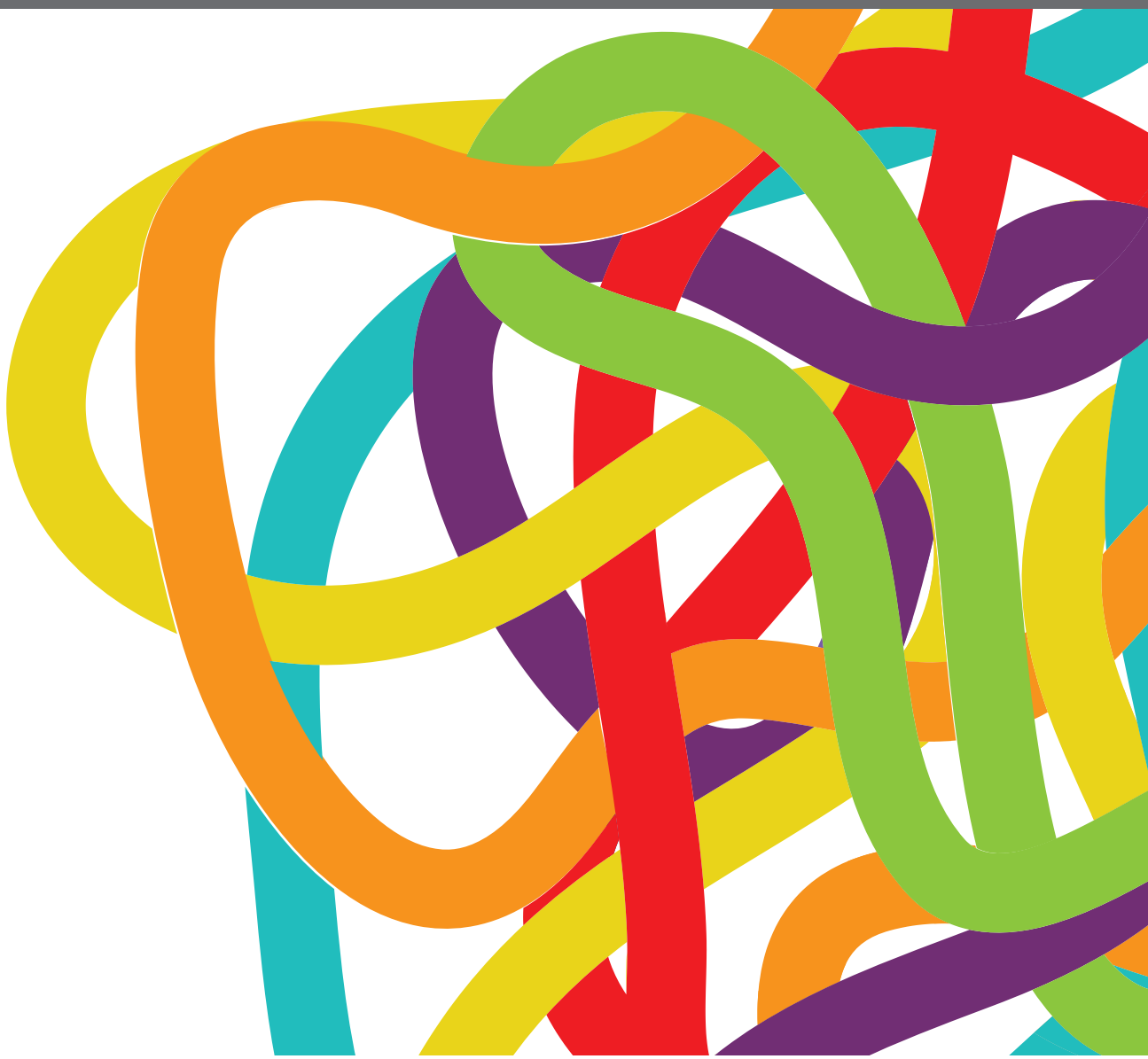
NEW ROLES OF AUTOPHAGY PATHWAYS IN CANCER

EDITED BY: Daniel Hector Grasso, Eugenia Morselli, Waleska Kerllen Martins and Claudio Marcelo Fader

PUBLISHED IN: Frontiers in Oncology and Frontiers in Cell and Developmental Biology



frontiers Research Topics

Frontiers eBook Copyright Statement

The copyright in the text of individual articles in this eBook is the property of their respective authors or their respective institutions or funders. The copyright in graphics and images within each article may be subject to copyright of other parties. In both cases this is subject to a license granted to Frontiers.

The compilation of articles constituting this eBook is the property of Frontiers.

Each article within this eBook, and the eBook itself, are published under the most recent version of the Creative Commons CC-BY licence. The version current at the date of publication of this eBook is CC-BY 4.0. If the CC-BY licence is updated, the licence granted by Frontiers is automatically updated to the new version.

When exercising any right under the CC-BY licence, Frontiers must be attributed as the original publisher of the article or eBook, as applicable.

Authors have the responsibility of ensuring that any graphics or other materials which are the property of others may be included in the CC-BY licence, but this should be checked before relying on the CC-BY licence to reproduce those materials. Any copyright notices relating to those materials must be complied with.

Copyright and source acknowledgement notices may not be removed and must be displayed in any copy, derivative work or partial copy which includes the elements in question.

All copyright, and all rights therein, are protected by national and international copyright laws. The above represents a summary only. For further information please read Frontiers' Conditions for Website Use and Copyright Statement, and the applicable CC-BY licence.

ISSN 1664-8714
ISBN 978-2-88971-281-6
DOI 10.3389/978-2-88971-281-6

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

NEW ROLES OF AUTOPHAGY PATHWAYS IN CANCER

Topic Editors:
Daniel Hector Grasso, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
Eugenia Morselli, Pontificia Universidad Católica de chile, Chile
Waleska Kerllen Martins, Universidade Anhanguera de São Paulo, Brazil
Claudio Marcelo Fader, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

Citation: Grasso, D. H., Morselli, E., Martins, W. K., Fader, C. M., eds. (2021). New Roles of Autophagy Pathways in Cancer. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88971-281-6

Table of Contents

05 ***Editorial: New Roles of Autophagy Pathways in Cancer***
Waleska K. Martins, Claudio M. Fader, Eugenia Morselli and Daniel Grasso

08 ***Significant Prognostic Value of the Autophagy-Related Gene P4HB in Bladder Urothelial Carcinoma***
Lei Lyu, Wei Xiang, Fuxin Zheng, Tao Huang, Yan Feng, Jingdong Yuan and Chuanhua Zhang

24 ***Comparison of Stage 4 and Stage 4s Neuroblastoma Identifies Autophagy-Related Gene and LncRNA Signatures Associated With Prognosis***
Xinyao Meng, Honglin Li, Erhu Fang, Jiexiong Feng and Xiang Zhao

41 ***Multifaceted WNT Signaling at the Crossroads Between Epithelial-Mesenchymal Transition and Autophagy in Glioblastoma***
Bárbara Paranhos Coelho, Camila Felix de Lima Fernandes, Jacqueline Marcia Boccacino, Maria Clara da Silva Souza, Maria Isabel Melo-Escobar, Rodrigo Nunes Alves, Mariana Brandão Prado, Rebeca Piatniczka Iglesia, Giovanni Cangiano, Giulia La Rocca Mazzaro and Marilene Hohmuth Lopes

58 ***Emerging Autophagy Functions Shape the Tumor Microenvironment and Play a Role in Cancer Progression - Implications for Cancer Therapy***
Silvina Odete Bustos, Fernanda Antunes, Maria Cristina Rangel and Roger Chammas

75 ***The Role of Autophagy in Tumor Immunology—Complex Mechanisms That May Be Explored Therapeutically***
Alana Serrano Campelo de Souza, Letícia Boslooper Gonçalves, Ana Paula Lepique and Patrícia Savio de Araujo-Souza

87 ***Deciphering the Role of the Coagulation Cascade and Autophagy in Cancer-Related Thrombosis and Metastasis***
Charlotte Nicole Hill, Maria Paz Hernández-Cáceres, Catalina Asencio, Begoña Torres, Benjamin Solis and Gareth I. Owen

112 ***Role of Autophagy in the Microenvironment of Oral Squamous Cell Carcinoma***
Daniel Peña-Oyarzún, Montserrat Reyes, María Paz Hernández-Cáceres, Catalina Kretschmar, Eugenia Morselli, Cesar A. Ramirez-Sarmiento, Sergio Lavandero, Vicente A. Torres and Alfredo Criollo

123 ***A Novel Autophagy-Related lncRNAs Signature for Prognostic Prediction and Clinical Value in Patients With Pancreatic Cancer***
Zhengdong Deng, Xiangyu Li, Yuanxin Shi, Yun Lu, Wei Yao and Jianming Wang

140 ***Autophagy and Exosomes Relationship in Cancer: Friends or Foes?***
Marta Colletti, Donatella Ceglie, Angela Di Giannatale and Francesca Nazio

151 ***Autophagy Regulation and Photodynamic Therapy: Insights to Improve Outcomes of Cancer Treatment***
Waleska K. Martins, Renata Belotto, Maryana N. Silva, Daniel Grasso, Maynne D. Suriani, Tayná S. Lavor, Rosangela Itri, Mauricio S. Baptista and Tayana M. Tsubone

173 ***Metformin Resensitizes Sorafenib-Resistant HCC Cells Through AMPK-Dependent Autophagy Activation***
Hong-Yue Lai, Hsin-Hwa Tsai, Chia-Jui Yen, Liang-Yi Hung, Ching-Chieh Yang, Chung-Han Ho, Hsin-Yin Liang, Feng-Wei Chen, Chien-Feng Li and Ju-Ming Wang

187 ***Pro-Tumoral Functions of Autophagy Receptors in the Modulation of Cancer Progression***
Cristóbal Cerda-Troncoso, Manuel Varas-Godoy and Patricia V. Burgos

200 ***Chaperone Mediated Autophagy Substrates and Components in Cancer***
Javiera Rios, Alvaro Sequeida, Amelina Albornoz and Mauricio Budini

209 ***Autophagy-Mediated Exosomes as Immunomodulators of Natural Killer Cells in Pancreatic Cancer Microenvironment***
Daniela L. Papademetrio, María Noé Garcia, Daniel Grasso and Élida Alvarez

218 ***Targeting Cytoprotective Autophagy to Enhance Anticancer Therapies***
Malina Xiao, Alice Benoit, Meriem Hasmim, Caroline Duhem, Guillaume Vogin, Guy Berchem, Muhammad Zaeem Noman and Bassam Janji

231 ***Mechanobiology of Autophagy: The Unexplored Side of Cancer***
Maria Paz Hernández-Cáceres, Leslie Munoz, Javiera M. Pradenas, Francisco Pena, Pablo Lagos, Pablo Aceiton, Gareth I. Owen, Eugenia Morselli, Alfredo Criollo, Andrea Ravasio and Cristina Bertocchi

251 ***Mitophagy in Pancreatic Cancer***
Yangchun Xie, Jiao Liu, Rui Kang and Daolin Tang

260 ***Autophagy in Viral Development and Progression of Cancer***
Alejandra Suares, María Victoria Medina and Omar Coso

289 ***Autophagy and Cancer Dormancy***
Yunus Akkoc, Nesibe Peker, Arzu Akcay and Devrim Gozuacik

311 ***Autophagy in Human T-Cell Leukemia Virus Type 1 (HTLV-1) Induced Leukemia***
Nicolás Ducasa, Daniel Grasso, Paula Benencio, Daniela L. Papademetrio, Mirna Biglione, Fatah Kashanchi, Carolina Berini and Maria Noé Garcia

320 ***Autophagy Process in Trophoblast Cells Invasion and Differentiation: Similitude and Differences With Cancer Cells***
Lorena Carvajal, Jaime Gutiérrez, Eugenia Morselli and Andrea Leiva

339 ***A Key Pathway to Cancer Resilience: The Role of Autophagy in Glioblastomas***
Elisa Helena Farias Jandrey, Marcelle Bezerra, Lilian Tiemi Inoue, Frank B. Furnari, Anamaria Aranha Camargo and Érico Tosoni Costa

354 ***Blockage of Extracellular Signal-Regulated Kinase Exerts an Antitumor Effect via Regulating Energy Metabolism and Enhances the Efficacy of Autophagy Inhibitors by Regulating Transcription Factor EB Nuclear Translocation in Osteosarcoma***
Man Zhang, Yang Bai, Chang Xu, Yiying Qi, Jiahong Meng, Wenkan Zhang, Hang Su and Weiqi Yan

frontiers in Oncology

EDITORIAL
published: 07 July 2021
doi: 10.3389/fonc.2021.726989

Editorial: New Roles of Autophagy Pathways in Cancer

Waleska K. Martins[1], *Claudio M. Fader*[2,3], *Eugenia Morselli*[4,5] *and Daniel Grasso*[6,7]*

[1] Laboratory of Cell and Membrane, Anhanguera University of Sao Paulo, São Paulo, Brazil, [2] Laboratorio de Biología Celular y Molecular, Instituto de Histología y Embriología (IHEM), Universidad Nacional de Cuyo, CONICET, Mendoza, Argentina, [3] Facultad de Odontología, Universidad Nacional de Cuyo, Mendoza, Argentina, [4] Laboratory of Autophagy and Metabolism, Department of Physiology, Faculty of Biological Sciences, Pontificia Universidad Católica De Chile, Santiago, Chile, [5] Autophagy Research Center, Santiago de Chile, Chile, [6] Instituto de Estudios de la Inmunidad Humoral (IDEHU), CONICET, Universidad de Buenos Aires, Buenos Aires, Argentina, [7] Departamento de Ciencias Biológicas, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina

Keywords: autophagy, cancer, research topic, molecular pathways, age-related disease

Editorial on the Research Topic

New Roles of Autophagy Pathways in Cancer

From a simplistic point of view, autophagy is a self-degradative process that relies on lysosomes for the removal of cytoplasmic bulk cargo and damaged organelles, such as mitochondria. Further on its homeostatic role, autophagy acts as a catabolic process that promotes cellular resilience in conditions of nutrient deprivation and energy depletion. A body of literature has established a crucial role of autophagy in a whole plethora of different physiological processes ranging from homeostasis maintenance, development, and differentiation, among others. In the last two decades, the complexity of autophagy regulation has grown exponentially. Indeed, the literature recognizes canonical and non-canonical autophagic pathways that lead to the degradation and clearance of non-specific or specific cargos (selective autophagy) depending on the cellular context. Due to the fundamental role of autophagy in homeostasis maintenance, it is not surprising its recognized etiologic role in age-related diseases, including cancer. In cancer, autophagy has a dual function, acting as a cell survival mechanism (e.g. favoring the growth of established tumors) or as a tumor suppressor (e.g. preventing the accumulation of damaged proteins and organelles). Thus, the relationship of autophagy with carcinogenesis is complex and, in most cases, it is considered a context-dependent process.

This collection compiles some of the most recent advances in the knowledge of the autophagic pathway and its involvement in human cancer development. Carcinogenesis implies proliferation, tissue invasion, vascularization, and modulation of the immune system. Herein, we expanded our knowledge about autophagy in carcinogenesis, showing how it has been engaged in various processes, including tumor progression, cancer-related thrombosis and metastasis, cancer dormancy linked to stem cell behavior and quiescence, epithelial-to-mesenchymal transition (EMT), intercellular communications, cell-stroma interactions, and tumor microenvironment (TME), immune responses, treatment resistance and tumor-adaptative response.

Interestingly, proliferation, invasion, vascularization, and immune modulation, which are characteristics of cancer, are also present in trophoblast cells during placentation. However,

OPEN ACCESS

Edited and reviewed by:
Kirill Afonin,
University of North Carolina at Charlotte, United States

***Correspondence:**
Daniel Grasso
dgrasso@ffyb.uba.ar

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *17 June 2021*
Accepted: *23 June 2021*
Published: *07 July 2021*

Citation:
Martins WK, Fader CM, Morselli E and Grasso D (2021) Editorial: New Roles of Autophagy Pathways in Cancer. Front. Oncol. 11:726989. doi: 10.3389/fonc.2021.726989

while these processes are strictly regulated during placentation, in the context of tumor growth the regulation is lost. Carvajal et al. discuss similarities and differences between carcinogenesis and placentation and the role of autophagy in the processes. As previously mentioned, autophagy can be a bulk process, or highly selective and Cerda-Troncoso et al. review the pro-tumorigenic roles of the better-described autophagy receptors such as p62/SQSTM1, NBR1, NDP52, and OPTN, which are overexpressed in cancer, and could be considered as new therapeutic targets against tumor growth. Additionally, Xie et al. describe the regulation of the autophagy-mediated selective turnover of mitochondria (mitophagy) in the highly mortal pancreatic ductal adenocarcinoma. Mitochondria quality control is critical for cell homeostasis and authors show the dual roles that the different mitophagy pathways (e.g., PINK-PRKN, BNIP3L/NIX, FUNDL1, and BNIP3) have in carcinogenesis and treatment of pancreatic cancer. Consistently, considering the different types of autophagy, Rios et al. analyze the contribution of chaperone-mediated autophagy in carcinogenesis. In an exciting emerging topic, Hernández-Cáceres et al. explore the involvement of cell mechanics during oncogenesis in autophagy regulation and its implication in disease progression. In response to the complex mechano-environment of tumor burden, cells activate mechanosensitive protein complexes and related-active cytoskeleton processes that impact the autophagy machinery and disease progression. On the other hand, instead of mechanical stimuli, autophagy can be regulated by external organisms such as viruses. Suares et al. and Ducasa et al. show how oncoviruses modulate the autophagy machinery enhancing viral survival and replication that eventually culminates in cell transformation. Altogether, these works suggest that a deeper and detailed comprehension of autophagy mechanisms might pave the way to explore precision therapy approaches against specific tumors.

Cancer is a tissular disease where cancerous cells are in dynamic communication with the different actors of TME and this relationship ultimately determines most of the tumor behavior. Consequently, many researchers focus their work to uncover the complexity of TME and its relationship with cancer cells. Coelho et al. describe the role of WNT signaling, in response to TME stress, in the regulation of the dichotomic fate between EMT and autophagy in glioblastoma. The autophagy pathway is also key for the TME establishment in oral squamous cell carcinoma, as described in Peña-Oyarzún et al. Furthermore, despite its actions over tumor cells, autophagy is also relevant for the communication among tumoral cells, TME, and the whole body. This autophagic role, as a means for intercellular communication, is highlighted in Bustos et al. where they describe the non-autophagic functions of the pathway, focusing on the new field of the autophagy-dependent secretion and its important implication for cancer fate (including the TME), the immune response and the biogenesis and secretion of extracellular vesicles (EVs). There seems to exist a strong relationship between the autophagy machinery and EVs, especially the exosomes that are EVs of endosomal origin. The state of the art concerning the autophagy-EVs complex associated with TME was investigated in Colletti et al. and Papademetrio et al. Noteworthy, the tumor-associated immune system is also reached by the plethora of different EVs of TME as it is shown in Colletti et al. and Papademetrio et al. Independently of the EVs, autophagy plays a key role in the tumor-associated immunology system, according to de Souza et al. The vast literature compiled in de Souza et al. reveals the key role of autophagy in tumor immunogenicity and how it engages TME, which might provide new insights into mitigating tumor relapse.

Beyond its pro-tumor role in carcinogenesis, autophagy highlights also as a promisor target for diagnosis and prognosis of cancer as discussed in this Research Topic. Meng et al., Lyu et al. and Deng et al. describe different autophagy-related genetic signatures as potential prognostic tools for neuroblastoma, bladder urothelial carcinoma, and pancreatic cancer, respectively. On the dark side, Akkoc et al. discussed autophagy's role in cancer dormancy, which eventually contributes to metastasis and relapse, the leading cause of cancer-related deaths. Thrombotic events due to an enhanced thrombotic state are a severe complication among patients suffering from different types of cancers. Interestingly, Hill et al. discuss autophagy´s role along with all the coagulation components system and highlights the related implications regarding the TME and tumor development. Moreover, it is well accepted that autophagy may elicit tumor therapeutic resistance, and thus, it has been considered as a pharmacologic target to alleviate tumor relapse. Towards this end, Lai et al. described how metformin might overcome the autophagy-mediated resistance to Sorafenib treatment in hepatocellular carcinoma patients. On the other hand, Xiao et al. discussed how to abrogate cytoprotective autophagy (i.e., by genetic and pharmacologic means) to improve antitumor therapeutic approaches. Similarly, Jandrey et al., based on compiling articles, contributed to deciphering the cancer resilience of glioblastoma cells to conventional chemotherapies in terms of the pro-survival autophagy proficiency. Furthermore, Martins et al. reveal how photodynamic therapy (PDT) mediated-oxidative stress may induce autophagy in tumor cells, and how lysosomal photodamage might trigger autophagy as a regulated cell death mechanism, improving the clinical outcome of PDT-treated patients.

Carcinogenesis is an age-related process, and as humans extend their life expectancy, the incidence of cancer will continue to increase. Although lots of progress has been made in the diagnosis and treatment of many human cancers, we are still far from a panacea. Thus, morbidity and mortality related to cancer remain a global issue. The milestone contributions presented in this Research Topic regarding the "new roles of the autophagy pathway in cancer" reveal the great advancement made in the underlying molecular mechanism of autophagy and its implication for the comprehension of cancer. Most works unveil an intricate and complex role of autophagy in tumoral cells, TME, and even the communications among them. Altogether, this knowledge provides us a glimpse of new therapeutic options to be explored, mainly in cancer resilience.

Furthermore, since autophagy in cancer is known to be a double edge sword, it is of great importance to get a deep insight of its role during carcinogenesis, to design specific therapeutic approaches. Finally, it is an exciting time of great discoveries about the autophagic pathway, and more importantly, those discoveries bring hope and assure great benefits in the long fight against a devastating disease like cancer.

AUTHOR CONTRIBUTIONS

WM, CF, EM, and DG contributed equally to this Research Topic by acting as guest editors, with professionalism, sharing their thoughts and experience, working in a group, and writing the editorial. All authors contributed to the article and approved the submitted version.

FUNDING

DG receives financial support from University of Buenos Aires (UBACyT 2020—20020190200047BA) and the *Agencia Nacional de Promoción Científica y Técnica* (ANPCyT—PICT-2018-02220).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Martins, Fader, Morselli and Grasso. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Oncology

ORIGINAL RESEARCH
published: 13 August 2020
doi: 10.3389/fonc.2020.01613

Significant Prognostic Value of the Autophagy-Related Gene P4HB in Bladder Urothelial Carcinoma

Lei Lyu[1]†, Wei Xiang[1]†, Fuxin Zheng[1]†, Tao Huang[1], Yan Feng[2], Jingdong Yuan[1] and Chuanhua Zhang[1]**

[1] Department of Urology, Wuhan No.1 Hospital, Huazhong University of Science and Technology, Wuhan, China,
[2] Department of Pathology, Wuhan No.1 Hospital, Huazhong University of Science and Technology, Wuhan, China

OPEN ACCESS

Edited by:
Claudio Marcelo Fader,
Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

Reviewed by:
Xiangqian Guo,
Henan University, China
Daniel Hector Grasso,
Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

***Correspondence:**
Lei Lyu
teecars07@hotmail.com
Chuanhua Zhang
CH_Zhang07@163.com

† These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: 13 May 2020
Accepted: 24 July 2020
Published: 13 August 2020

Citation:
Lyu L, Xiang W, Zheng F, Huang T, Feng Y, Yuan J and Zhang C (2020) Significant Prognostic Value of the Autophagy-Related Gene P4HB in Bladder Urothelial Carcinoma. Front. Oncol. 10:1613. doi: 10.3389/fonc.2020.01613

While hundreds of consistently altered autophagy-related genes (ARGs) have been identified in cancers, their prognostic value in bladder urothelial carcinoma (BUC) remains unclear. In the present study, we collected 232 ARGs from the Human Autophagy Database (HADb), and identified 37 differentially expressed ARGs in BUC based on The Cancer Genome Atlas (TCGA) database. Kaplan-Meier survival analysis based on the Gene Expression Profiling Interactive Analysis (GEPIA) database revealed that among the 37 differentially expressed ARGs, prolyl 4-hydroxylase, beta polypeptide (P4HB), and regulator of G protein signaling 19 (RGS19) were significantly negatively correlated with overall survival (OS) and disease-free survival (DFS). Overexpression of P4HB and RGS19 in BUC was further validated using independent data sets, including those from the Oncomine and Gene Expression Omnibus (GEO) databases. cBioPortal and UALCAN analyses indicated that altered P4HB and RGS19 mRNA expression was significantly associated with mutations and clinical characteristics (nodal metastasis and cancer stage). Moreover, co-expression network analysis and gene set enrichment analysis (GSEA) predicted that the potential functions of P4HB and RGS19 are involved in the endoplasmic reticulum (ER) stress response, cytokine-mediated signaling pathway and inflammatory response. More importantly, multivariate Cox proportional hazards regression analysis demonstrated that P4HB, but not RGS19, is an independent and unfavorable BUC biomarker based on clinical characteristics (age, gender, cancer stage, and pathological TNM stage). Finally, we validated that the mRNA and protein expression levels of P4HB were upregulated in four bladder cancer cell lines (T24, J82, EJ, and SW780) and found that knockdown of P4HB dramatically inhibited the invasion and proliferation of bladder cancer cells. In summary, our study screened ARGs and identified P4HB as a biomarker that can predict the progression and prognosis of BUC and may provide a better understanding of the autophagy regulatory mechanisms involved in BUC.

Keywords: bladder urothelial carcinoma, autophagy-related genes, prognosis, biomarker, prolyl 4-hydroxylase, beta polypeptide

INTRODUCTION

Bladder cancer is the most common malignancy of the urinary system and the leading cause of cancer-associated mortality in the elderly population of China (1). The pathological types of bladder cancer mainly include bladder urothelial carcinoma (BUC), bladder squamous cell carcinoma and bladder adenocarcinoma. The most common pathological type of bladder cancer is BUC, which has unique characteristics, such as drug resistance, a high recurrence rate, a higher frequency of metastasis, and poor prognosis (2, 3). However, traditional clinicopathological risk factors could not sufficiently identify BUC patients with high risk and predict the prognosis of BUC. Recently, molecular biomarkers have been shown to aid the diagnosis and therapy and guide the prediction of the prognosis for BUC (4). For example, Chang et al. reported that BCAT1 is a potential diagnostic and prognostic marker for BUC patients (5). Zhang et al. revealed that high expression of HEF1 is associated with advanced stage and shortened progression-free survival poor for BUC patients (6). However, the clinical significance of these potential biomarkers and functionally important genes were not definitively verified because of a lack of larger clinical cohorts. Therefore, it is necessary to identify valuable biomarkers using large clinical samples and further investigate the molecular mechanisms involved in the development of BUC.

Currently, autophagy has been extensively studied and proposed as a significant factor in tumor development (7–9). Autophagy has both protective and harmful biological functions, including pro-, or antitumor effects, depending on the tumor microenvironment. On the one hand, autophagy enhanced cancer cells to tolerate stress responses, including a hypoxic microenvironment, starvation, and chemotherapy (10, 11). On the other hand, autophagy plays a critical role in damage mitigation in response to stress that can inhibit tumor progression by degrading defective proteins and organelles and by preventing abnormal protein accumulation (12). Regarding BUC, autophagy-targeted therapy has recently been considered a valuable strategy. It was recently reported that autophagy-related gene (ARG) levels are associated with the chemosensitivity of BUC and markedly affect the regulation of invasion (13). However, the role and mechanisms of autophagy remain far from clear. In the present study, we used bioinformatics analysis to investigate the expression of variations in 223 ARGs in BUC and to explore their potency as biomarkers. We finally identified prolyl 4-hydroxylase, beta polypeptide (P4HB) as a novel potential biomarker for BUC diagnosis and prognosis. Moreover, we demonstrated that knockdown of P4HB in human bladder cancer cells *in vitro* dramatically inhibited cancer cell invasion. The present study developed an ARG into a potential biomarker that provides a deeper understanding of the mechanism of autophagy in BUC.

MATERIALS AND METHODS

Collection of ARGs

We first collected 232 ARGs from the Human Autophagy Database (HADb[1]). Subsequently, we downloaded the RNA expression profiles (RNA-Seq2 level 3 data; platform: Illumina HiSeq 2000, through Mar 2020) and clinical data of BUC patients from The Cancer Genome Atlas (TCGA) database[2]. TCGA provided the mRNA expression data of 430 samples (411 BUC samples and 19 normal bladder tissue samples).

Functional Enrichment Analysis

The GO database[3] was used to analyze differentially expressed ARGs. The molecular functions (MFs), cellular components (CCs), and biological processes (BPs) of differentially expressed ARGs were identified. The potential functions of the differentially expressed ARGs involved in signaling pathways were analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG)[4] and Reactome[5]. The relationships among the enriched clusters from the GO and signaling pathway analyses were visualized using Metascape[6] (14). In addition, gene set enrichment analysis (GSEA) was performed to evaluate the correlation between P4HB or regulator of G protein signaling 19 (RGS19) expression and BUC-associated pathways. The detailed protocol for GSEA is available on the Broad Institute Gene Set Enrichment Analysis website[7] (15).

Survival Analysis

Kaplan-Meier survival curves were generated to evaluate the prognostic value of the ARGs using the online database Gene Expression Profiling Interactive Analysis (GEPIA2)[8] (16). For the overall survival (OS) and disease-free survival (DFS) analyses, the BUC patients were divided into 2 groups according to the median expression of the mRNAs (high vs. low). The survival curves of samples with low mRNA expression and high mRNA expression were compared using the log-rank test. $P < 0.05$ indicated statistically significant differences. In addition, multivariate Cox proportional hazards regression analysis was performed to determine the P4HB, RGS19, and clinical features that were significantly associated with OS.

Oncomine Database and GEO Database Analyses

The mRNA expression of P4HB and RGS19 in BUC was analyzed within the Oncomine database[9]. The thresholds were restricted as follows: *P*-value: 0.0001, fold change: 1.5, gene ranking: all, analysis type: cancer vs. normal, and data type: mRNA. This

[1]http://www.autophagy.lu
[2]https://portal.gdc.cancer.gov
[3]http://www.geneontology.org
[4]http://www.kegg.jp
[5]https://reactome.org
[6]http://www.metascape.org/
[7]http://www.broad.mit.edu/gsea
[8]http://gepia.cancer-pku.cn
[9]www.oncomine.org

analysis drew on a series of BUC studies, including the Modlich, Sanchez-Carbayo, Dyrskjot and Lee studies (17–20). In addition, the mRNA expression of P4HB and RGS19 was validated in three independent Gene Expression Omnibus (GEO)[10] data sets (GSE13507, GSE52519, and GSE37815) using GEO2R. The gene expression profiling databases were obtained from GEO.

UALCAN and cBioPortal Analyses

UALCAN is an interactive web portal for facilitating tumor subgroup gene expression and survival analyses[11] (21). We used UALCAN analysis to estimate the P4HB and RGS19 expression levels based on the clinical features (gender, age, cancer stage, and nodal metastasis status) of BUC from TCGA data sets. The cBioPortal for cancer genomics[12] is an exploratory analysis tool for exploring multidimensional cancer genomics data sets. The frequency of P4HB and RGS19 alterations (amplification, deep deletion, and missense mutation) and copy number variations (CNVs) were obtained from Genomic Identification of Significant Targets in Cancer (CISTC). In addition, the OncoPrint sub-tool of cBioPortal was also utilized to analyze the integrated status of CNVs for P4HB and RGS19.

LinkedOmics Analysis

LinkedOmics[13] is a publicly available portal that includes multiomics data from all 32 TCGA cancer types (22). The LinkFinder module of LinkedOmics was used to analyze the differentially expressed genes correlated with P4HB or RGS19 in BUC from TCGA cohort. We constructed a co-expression network based on the Pearson correlation coefficient ($|\ cor| > 0.35$, $P < 0.05$) between P4HB or RGS19 and the mRNAs to predict the potential targets of P4HB or RGS19. In addition, we used GeneMANIA[14] (23) to visualize the gene network of P4HB and RGS19.

Cell Culture

Three human BUC lines (T24, J82, and SW780) and a human normal uroepithelial cell line (SV-HUC-1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, United States). The BUC EJ cell line was obtained from the Institute of Biochemistry and Cell Biology of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Thermo Scientific HyClone, Logan, UT, United States), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO_2.

RNA Interference and Transfection

The mRNA sequence of the P4HB gene was obtained from GenBank (NM_000918), and the targeting sequence was designed using an RNAi algorithm available online[15]. The P4HB-specific siRNA (5′-GTCCTCTTTAAGAAGTTTGATGA-3′) and a nonsense siRNA [negative control siRNA (NC siRNA)] were synthesized and purified by GenePharma (Shanghai, China). T24 and EJ cells were transfected with siRNAs using Translipid reagent (TransGen, Beijing, China) according to the manufacturer's protocol.

qRT-PCR

Total RNA was extracted using the TRIzol Reagent kit (Invitrogen, Carlsbad, CA, United States) and was reverse transcribed into cDNA by using PrimeScript RT-polymerase (TaKaRa, Dalian, China). Real-time PCR was performed on the cDNA templates using specific primers (Sangon, Shanghai, China) and SYBR master mix (TaKaRa, Dalian, China). The relative mRNA expression levels of P4HB were calculated as a ratio normalized to GAPDH expression. Comparative quantification was performed using the $2^{-\Delta\ \Delta\ Ct}$ method. The sequences of the specific primers used in the present study were as follows: P4HB (NM_000918), forward primer 5′-TCACATCCTGCTGTTCTTG-3′, reverse primer 5′-ACTTGGTCATCTCCTCCTC-3′; and GAPDH (NM_002046), forward primer 5′-TGAAGGTCGGAGTCAACGG-3′, and reverse primer 5′-CCTGGAAGATGGTGATGGG-3′.

Western Blot

Bladder urothelial carcinoma tissues and cells were lysed with RIPA buffer containing protease inhibitor (Thermo Fisher Scientific, Waltham, MA, United States). Proteins were quantified and resolved by 12% SDS-PAGE and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore Bedford, MA, United States). Then, the cells were incubated with 5% skim milk at room temperature for 30 min and with primary antibodies against P4HB (Cell Signaling Technology, Beverly, MA, United States) overnight at 4°C. Then, the cells were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz, CA, United States) and detected using a chemiluminescence method (ECL, Thermo Fisher Scientific, Waltham, MA, United States) according to the manufacturer's instructions. An anti-GAPDH (Santa Cruz, United States) antibody was used as a control.

Cell Viability Assays

The effect of P4HB-specific siRNA on cell viability was tested using an MTT assay (Beyotime Institute Biotechnology, Shanghai, China) according to the manufacturer's instructions. Briefly, T24 and EJ cells at 5×10^3 cells per well were cultured in 96-well plates and transfected with 100 nM NC siRNA and P4HB-specific siRNA for the indicated periods. Then, 20 μl MTT (5 mg/ml) was added to each well, and the cells were incubated for an additional 4 h. The relative levels of cell proliferation in each group of cells compared to that in control cells were calculated, and the control cells were designated to have a cell proliferation rate of 100%. All experiments were repeated at least three times.

Invasion Assays

Cell invasion assays were performed in a Boyden chemotaxis chamber (Costar, United States). Briefly, 5×10^4 cells

[10] www.ncbi.nlm.nih.gov/gds

[11] http://ualcan.path.uab.edu/analysis.html

[12] http://cbioportal.org

[13] http://www.linkedomics.org/login.php

[14] http://genemania.org

[15] http://sidirect2.rnai.jp

resuspended in serum-free RPMI 1640 medium were placed in the upper chamber, while the lower chamber was filled with 10% FBS-containing RPMI 1640. After incubation for 24 h, the cells in the upper chamber were removed, and the cells at the bottom of the polycarbonate membrane were fixed and stained with 0.1% crystal violet. The number of invading cells was counted in three randomly chosen fields under the microscope.

RESULTS

Differentially Expressed ARGs in TCGA

A total of 232 ARGs were collected from the HADb. The expression level of each of the 232 ARGs was compared between BUC and normal bladder tissues in the TCGA dataset, which contained 411 BUC samples and 19 adjacent non-tumor bladder tissue samples. Thirty-seven differentially expressed ARGs were identified, among which 18 were upregulated and 19 were downregulated (**Figures 1A,B**). All of the differentially expressed genes are listed in **Table 1**.

Enrichment Analyses of Differentially Expressed ARGs

We performed GO and KEGG pathway enrichment analyses to determine the potential functions of these dysregulated ARGs in the development of BUC. The GO plot analysis indicated that in the BPs, these genes were associated with the intrinsic apoptotic signaling pathway, as well as with the response to ER stress and the cell cycle. In terms of the CCs, these genes were involved in the autophagosome, mitochondrial outer membrane and cell adhesion. With regard to MF, these genes participated in certain key functions, such as ubiquitin protein ligase binding and protein phosphatase binding. The significant KEGG pathways in which the differentially expressed ARGs were enriched were mainly the p53 signaling pathway, apoptosis, autophagy and PI3K-Akt signaling pathway. In addition, the KEGG pathway enrichment analysis indicated that these genes were associated with multiple cancers, such as bladder cancer, pancreatic cancer, chronic myeloid leukemia, and breast cancer, which identified the major roles of these genes in tumorigenesis and development (**Figures 1C,D**).

Kaplan-Meier Survival Analyses of Differentially Expressed ARGs

To explore whether the differentially expressed ARGs were correlated with survival time, BUC cases were divided into two groups (low vs. high groups) according to the expression level of ARGs, and each group was analyzed by Kaplan-Meier survival analysis using the GEPIA database. The results showed

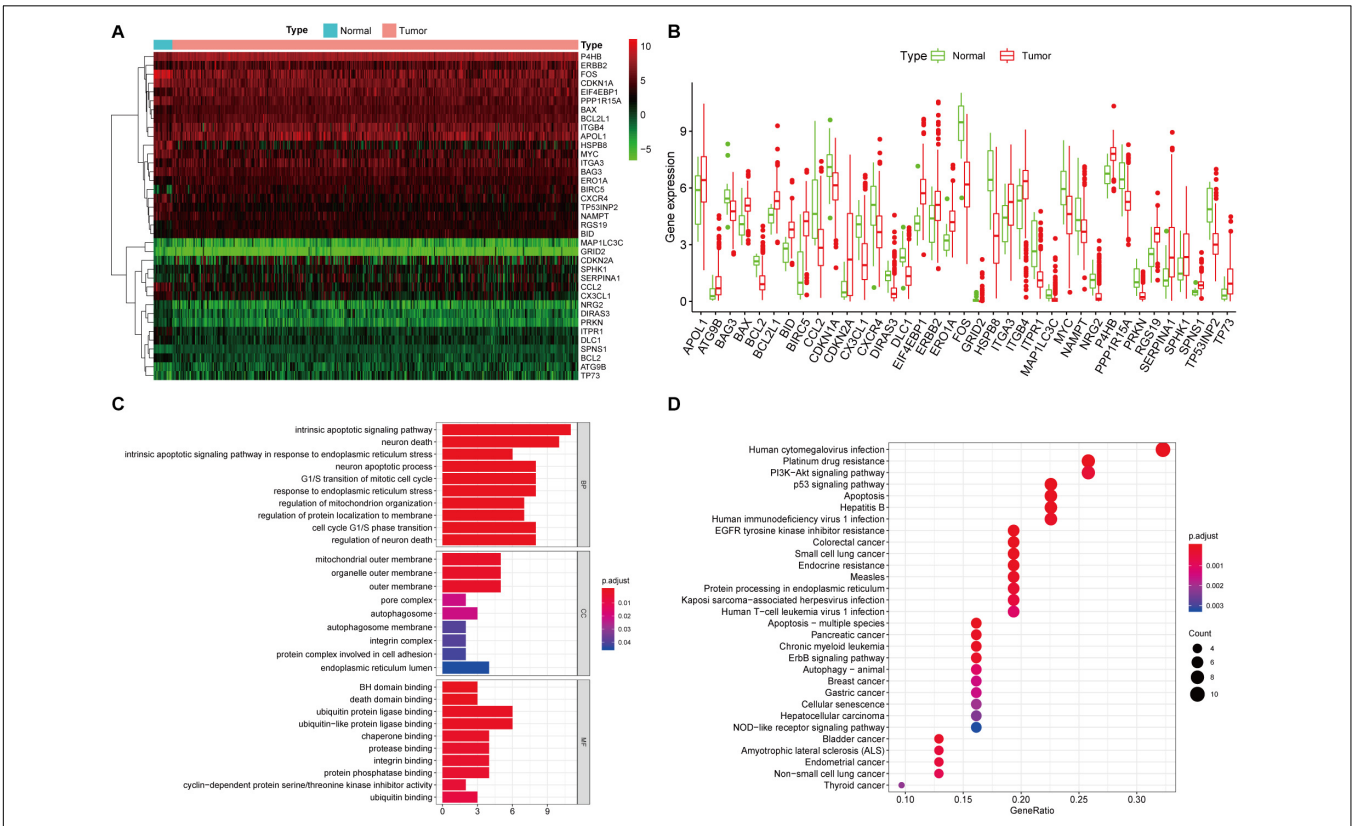


FIGURE 1 | Differentially expressed ARGs and functional annotation. **(A)** Heatmap of the differentially expressed ARGs. **(B)** The differentially expressed ARGs exhibited as a histogram. **(C)** GO enrichment analysis of differentially expressed ARGs. **(D)** KEGG pathway enrichment analysis of differentially expressed ARGs. The top 30 enriched pathways are shown.

TABLE 1 | The differentially expressed ARGs in BUC (Tumor vs. Normal).

Gene symbol	Ensembl ID	logFC	Regulation	*P*-value	FDR
CDKN2A	ENSG00000147889	4.19	Up	2.49E-03	5.39E-03
SERPINA1	ENSG00000197249	2.85	Up	1.16E-03	3.12E-03
BIRC5	ENSG00000089685	2.09	Up	8.84E-09	1.59E-07
TP73	ENSG00000078900	1.96	Up	2.51E-04	8.50E-04
ATG9B	ENSG00000181652	1.70	Up	8.09E-04	2.26E-03
EIF4EBP1	ENSG00000187840	1.68	Up	1.27E-09	3.51E-08
SPHK1	ENSG00000176170	1.52	Up	1.64E-02	2.96E-02
ERBB2	ENSG00000141736	1.45	Up	3.68E-03	7.73E-03
BID	ENSG00000015475	1.35	Up	7.39E-10	2.85E-08
ITGA3	ENSG00000005884	1.22	Up	4.54E-03	9.13E-03
APOL1	ENSG00000100342	1.18	Up	2.55E-02	4.28E-02
RGS19	ENSG00000171700	1.16	Up	2.44E-07	2.24E-06
ERO1A	ENSG00000197930	1.10	Up	3.07E-07	2.58E-06
P4HB	ENSG00000185624	1.09	Up	1.19E-09	3.51E-08
SPNS1	ENSG00000169682	1.09	Up	1.68E-08	2.50E-07
ITGB4	ENSG00000132470	1.06	Up	7.56E-04	2.16E-03
BCL2L1	ENSG00000171552	1.03	Up	5.10E-07	4.10E-06
BAX	ENSG00000087088	1.00	Up	9.04E-09	1.59E-07
MAP1LC3C	ENSG00000197769	−1.01	Down	4.41E-05	1.89E-04
GRID2	ENSG00000152208	−1.01	Down	8.41E-08	9.55E-07
BAG3	ENSG00000151929	−1.12	Down	1.05E-04	4.14E-04
NAMPT	ENSG00000105835	−1.15	Down	7.33E-03	1.39E-02
PPP1R15A	ENSG00000087074	−1.23	Down	5.54E-06	3.45E-05
CX3CL1	ENSG00000006210	−1.25	Down	2.01E-07	1.94E-06
CXCR4	ENSG00000121966	−1.27	Down	2.19E-03	4.92E-03
DIRAS3	ENSG00000162595	−1.33	Down	2.18E-08	3.01E-07
CDKN1A	ENSG00000124762	−1.35	Down	2.51E-04	8.50E-04
BCL2	ENSG00000171791	−1.36	Down	5.96E-10	2.85E-08
DLC1	ENSG00000164741	−1.40	Down	1.66E-07	1.68E-06
MYC	ENSG00000136997	−1.44	Down	1.71E-05	9.72E-05
NRG2	ENSG00000158458	−1.73	Down	9.04E-09	1.59E-07
TP53INP2	ENSG00000078804	−1.97	Down	1.90E-10	2.05E-08
ITPR1	ENSG00000150995	−2.40	Down	1.12E-08	1.80E-07
PRKN	ENSG00000185345	−2.43	Down	3.46E-10	2.23E-08
HSPB8	ENSG00000152137	−2.83	Down	2.91E-09	7.02E-08
CCL2	ENSG00000108691	−2.89	Down	1.57E-06	1.01E-05
FOS	ENSG00000170345	−2.94	Down	2.13E-10	2.05E-08

that among the 37 differentially expressed ARGs, only two ARGs (P4HB and RGS19) were significantly negatively correlated with OS and DFS (log-rank test, $P < 0.05$), suggesting that the expression levels of P4HB (**Figures 2A,B**), and RGS19 (**Figures 2C,D**) were closely related to BUC prognosis. In the following studies, we focused on investigating the biological role of these two genes in BUC.

Validation of the mRNA Expression of P4HB and RGS19 in the Oncomine and GEO Databases

To validate the difference in P4HB and RGS19 expression in tumor and normal tissues, especially in BUC, the P4HB and RGS19 mRNA levels in different tumors and normal tissues of multiple cancer types were analyzed using the Oncomine database. The analysis results revealed that P4HB expression was higher in bladder, brain, breast, kidney, lung, prostate, ovarian cancers, and lymphoma tumors than in normal tissues. RGS19 expression was higher in bladder, breast, and kidney cancer tissues than in normal tissues. However, expression of P4HB in esophageal, head, and neck cancers and leukemia was lower than expression in other cancers in some data sets. Moreover, expression of RGS19 was lower in leukemia, lung cancer, and lymphoma than in other cancers (**Figure 3A**). These results further confirmed that both the P4HB and RGS19 expression levels were higher in BUC than in normal bladder tissue (**Figures 3B,C**). The expression of P4HB and RGS19 was further tested in three independent GEO data sets (GSE13507, GSE52519, and GSE37815) using GEO2R. Consistent with

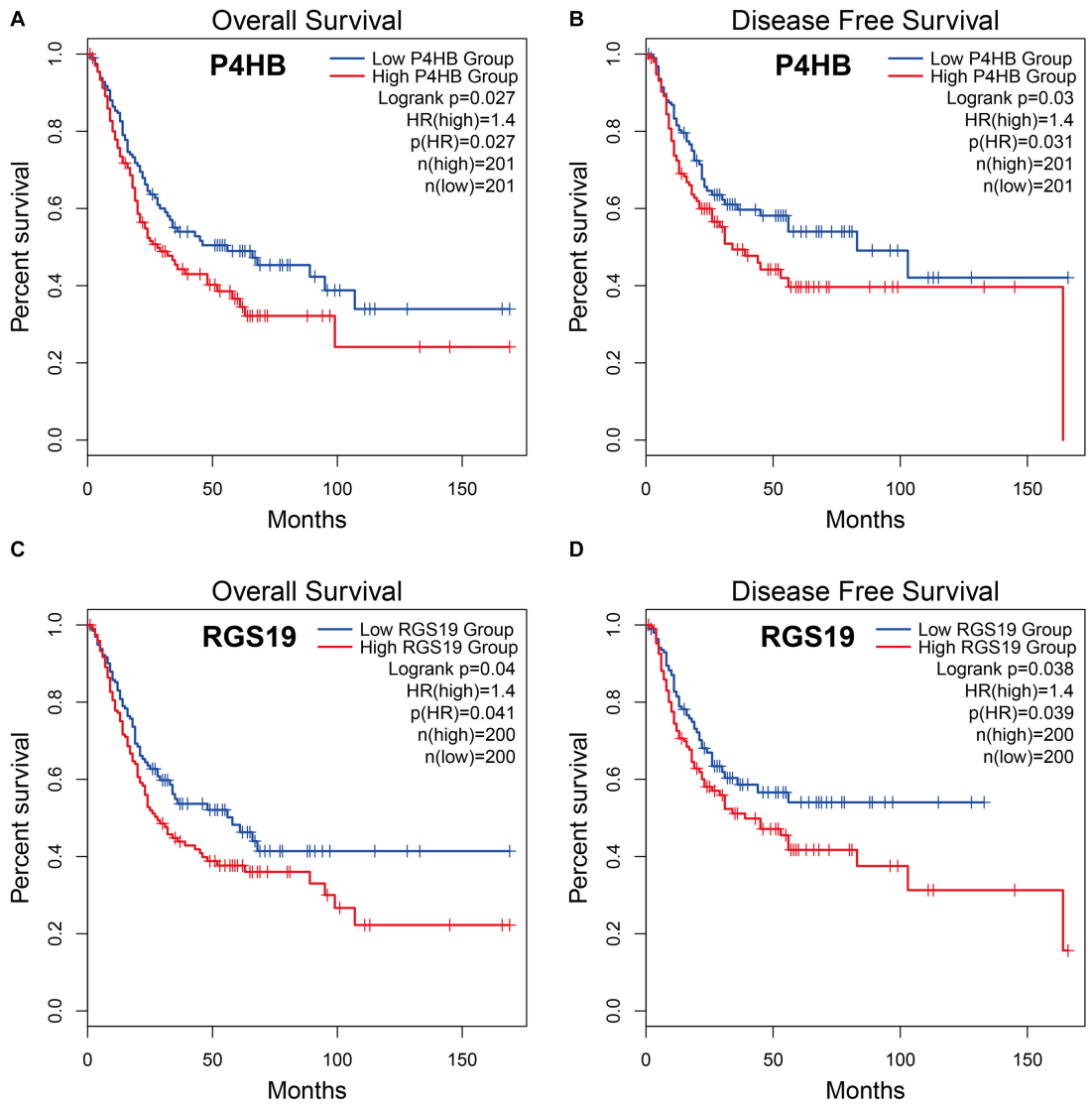


FIGURE 2 | Relationship between P4HB and RGS19 expression and prognosis in BUC patients. **(A,B)** Kaplan-Meier survival curves of OS and DFS comparing patients with high (red) and low (blue) expression of P4HB in BUC. **(C,D)** Kaplan-Meier survival curves of OS and DFS comparing patients with high (red) and low (blue) expression of RGS19 in BUC.

the results of TCGA database and Oncomine database, the mRNA expression levels of P4HB and RGS19 in BUC were upregulated compared with the expression levels in normal bladder tissue (**Table 2**).

Genomic Alterations of P4HB and RGS19 in BUC

We analyzed the genomic alterations of P4HB and RGS19 by using the cBioPortal online database for BUC. P4HB and RGS19 were altered in 52 (12.62%) and 25 (6%) of the 413 BUC patients, respectively. For P4HB, these alterations were mRNA upregulation in 30 cases (7.28%), mRNA downregulation in 3 cases (0.73%), amplification in 6 cases (1.46%), mutation in 5 cases (1.21%), and multiple alterations in 8 cases (1.94%). For RGS19, these alterations were mRNA upregulation in 17 cases (4.13%), amplification in 5 cases (1.21%), and mutation in 3 cases (0.73%; **Figures 4A,B**). Next, we analyzed the mutant mRNA expression of P4HB and RGS19. There was a significant difference in the P4HB and RGS19 expression levels between shallow deletion and amplification in the copy number alteration status in BUC, according to TCGA database analysis (**Figure 4C**).

Relationship Between the mRNA Levels of P4HB or RGS19 and the Clinicopathological Features of Patients With BUC

To elucidate the relevance of P4HB and RGS19 with respect to tumor progression, we further performed a subgroup analysis of

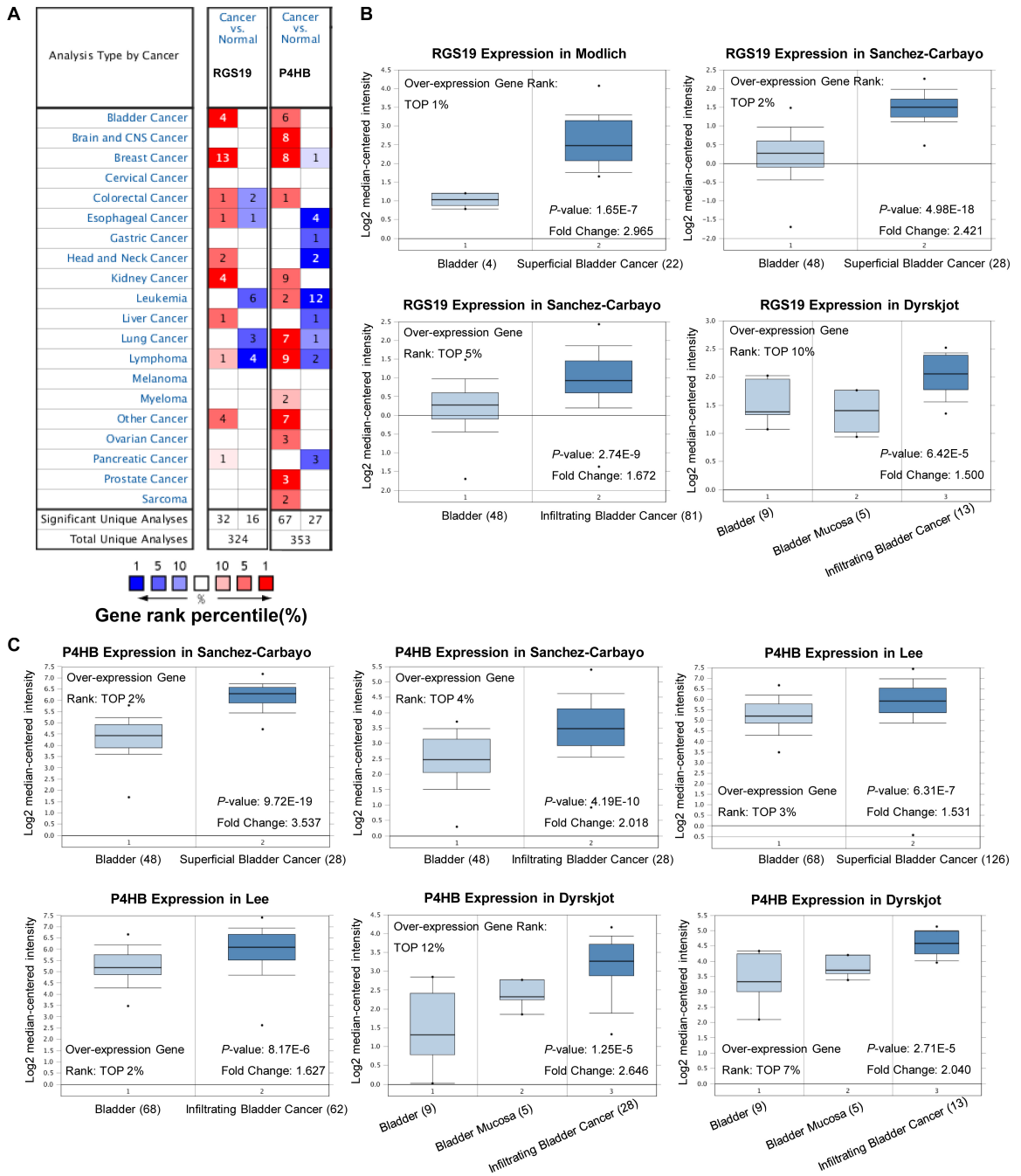


FIGURE 3 | Expression patterns of P4HB and RGS19 in the Oncomine database. **(A)** The transcription levels of P4HB and RGS19 in different types of cancers. **(B)** Box plot showing the RGS19 mRNA levels in the Modlich, Sanchez-Carbayo, and Dyrskjot bladder cancer data sets. **(C)** Box plot showing the P4HB mRNA levels in the Sanchez-Carbayo, Lee and Dyrskjot bladder cancer data sets.

multiple BUC clinicopathological features. The analysis included stage II–IV BUC cases (stage I was omitted due to only two cases being available from TCGA database). The transcription levels of P4HB and RGS19 were significantly higher in the tumor tissues than in the non-cancerous bladder tissues in subgroup analyses based on gender, age, cancer stage and nodal metastasis status (**Figures 5A,B**). In addition, we analyzed the protein expression levels of P4HB and RGS19 in BUC using The Human Protein Atlas (THPA) database[16]. The results revealed that the protein expression levels of P4HB in BUC tissue were also upregulated compared with those in normal bladder tissue. However, the

[16]https://www.proteinatlas.org

TABLE 2 | The mRNA expression of P4HB and RGS19 in the GEO databases.

Gene symbol	Ensembl ID	GEO	Platform	Platform ID	Fold change	*P* value
RGS19	ENSG00000171700	GSE13507	GPL6102	ILMN_1677085	1.26	3.56E-02
		GSE52519	GPL6884		2.15	4.36E-03
		GSE37815	GPL6102		1.24	1.30E-01
P4HB	ENSG00000185624	GSE13507	GPL6102	ILMN_1719303	1.89	3.31E-04
		GSE52519	GPL6884		1.75	2.36E-02
		GSE37815	GPL6102		1.98	8.07E-05

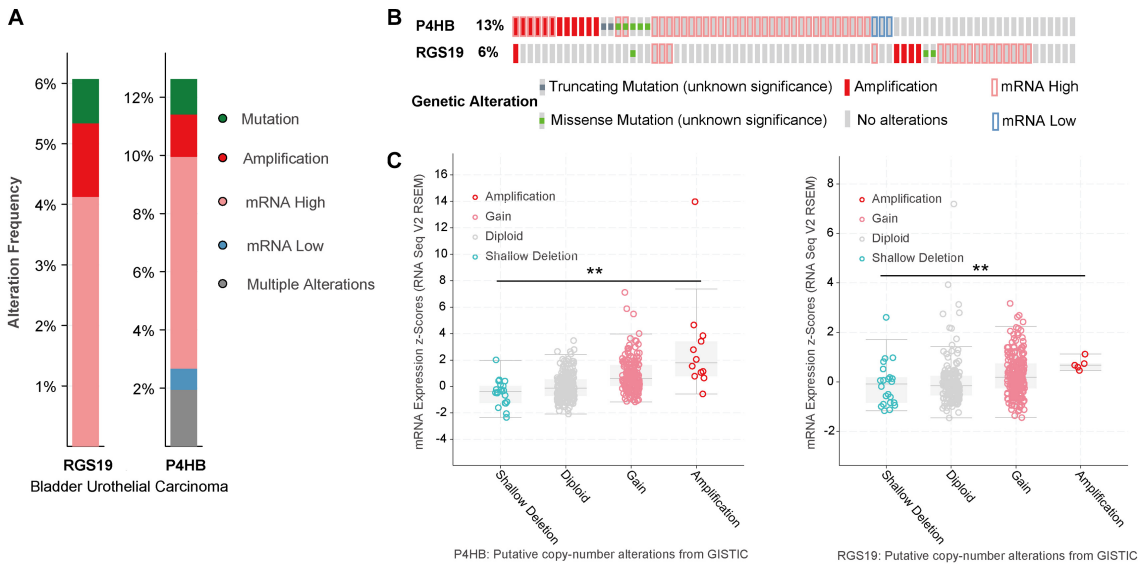


FIGURE 4 | Alteration frequency and mRNA expression of P4HB and RGS19 in BUC (cBioPortal). **(A)** Summary of alterations in P4HB and RGS19. **(B)** Oncoprint represents the distribution and proportion of samples with different types of alterations in P4HB and RGS19. **(C)** The relationship of P4HB or RGS19 mRNA expression and gene mutations. $^{**}P < 0.001$.

RGS19 protein was not detected in the BUC tissue or normal bladder tissue (**Figure 5C**).

Biological Interaction Network of P4HB and RGS19 in BUC

We first confirmed the biological functions of P4HB and RGS19 using the GeneMANIA database[17]. The analysis results showed that P4HB and RGS19 had been reported to be mainly associated with lipid metabolism and GTPase activity, respectively (**Figure 6**). Then, we predicted the potential functions of P4HB and RGS19 in BUC using co-expression analysis methods. The Function module of LinkedOmics was used to analyze the co-expressed genes correlated with P4HB or RGS19 from 413 BUC cases in TCGA. As shown in the volcano plot (**Figures 7A,B**), 1619 and 3160 genes (red dots) showed significant positive correlations with P4HB (top three genes: ANAPC11, STRA13, and SLC39A7) and RGS19 (top three genes: OPRL1, ARPC1B, and OGFR), respectively, whereas 1843 and 2788 genes (green dots) showed significant negative correlations [false discovery rate (FDR) < 0.01] with P4HB (top three genes:ZSWIM6, LYRM7, and WDR36) and RGS19 (top three genes: YTHDC1, TMTC2, and NHSL1), respectively. The 50 significant gene sets positively and negatively correlated with P4HB and RGS19, as shown in the heat map (**Figures 7C,D**). To obtain new insights into the potential functions of P4HB and RGS19, we performed co-expression network analysis based on the Pearson correlation coefficient ($|cor| \geq 0.30$, FDR < 0.01). A total of 276 and 620 protein-coding genes (PCGs) were highly correlated with P4HB and RGS19, respectively. Enrichment analyses based on GO terms, KEGG and Reactome pathways were performed to predict the potential functions of all PCGs correlated with P4HB and RGS19. The results showed that P4HB may be involved in autophagy, the response to endoplasmic reticulum (ER) stress and galactose metabolic processes. In addition, the potential functions of RGS19 may be associated with the immune response-regulating signaling pathway, cytokine-mediated signaling pathway, and inflammatory response (**Figure 8**). Subsequently, we performed GSEA using TCGA data to further validate the potential biological functions of P4HB and RGS19. Consistent with the results described above, stratified expression levels of P4HB and RGS19 were significantly correlated with genes associated with

[17]http://genemania.org

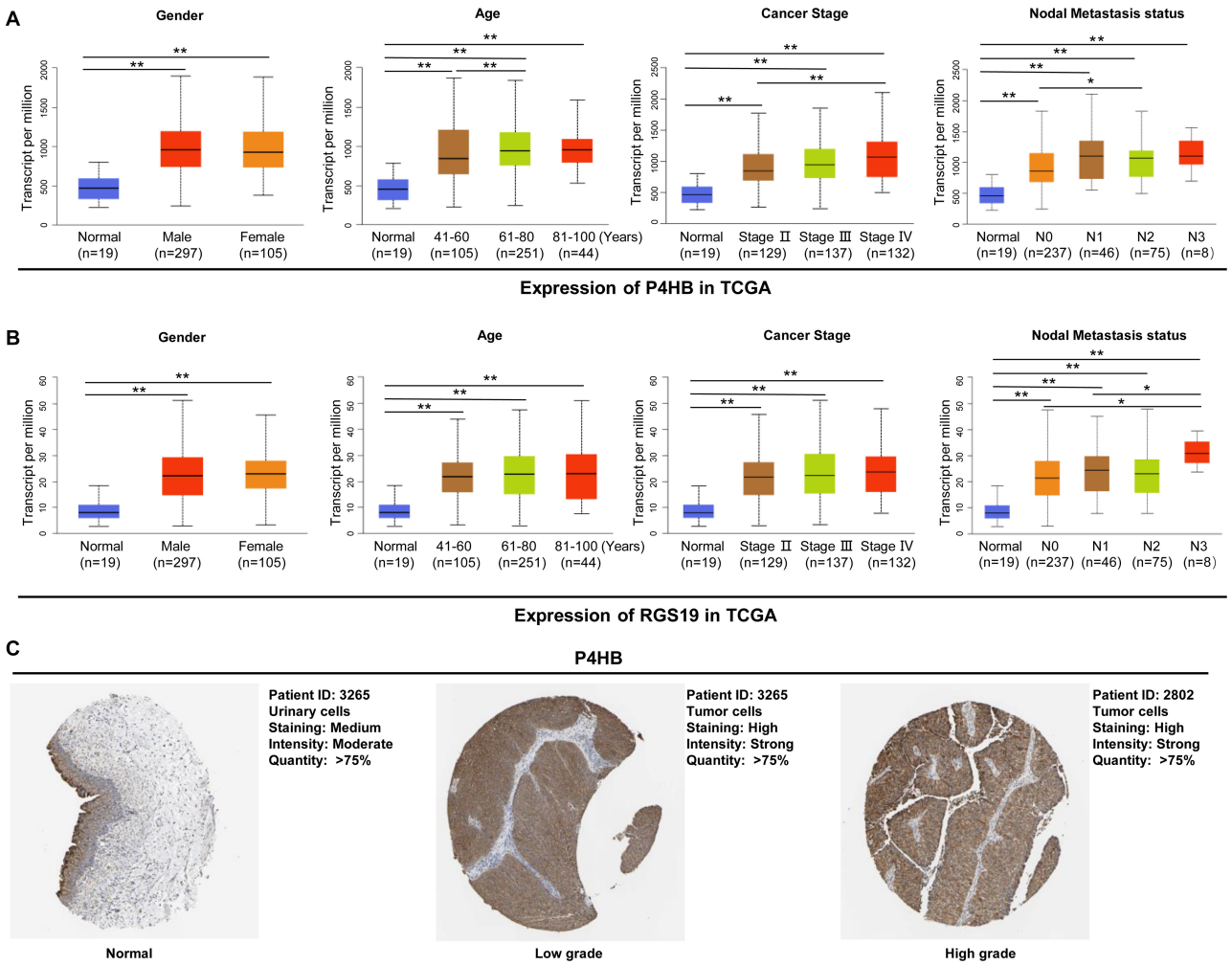


FIGURE 5 | P4HB and RGS19 mRNA and protein expression in subgroups of patients with BUC. Boxplot showing the relative expression of P4HB **(A)** and RGS19 **(B)** mRNA in normal bladder tissues and subgroups of BUC samples stratified based on gender, age, cancer stage, and nodal metastasis status. **(C)** The representative protein expression of P4HB in normal bladder tissues, low grade and high grade BUC. $^{**}P < 0.01$, $^{*}P < 0.05$.

the nucleotide sugar metabolic process, regulation of autophagy, cytokine-mediated signaling pathway, and immune response (**Figures 9A,B**).

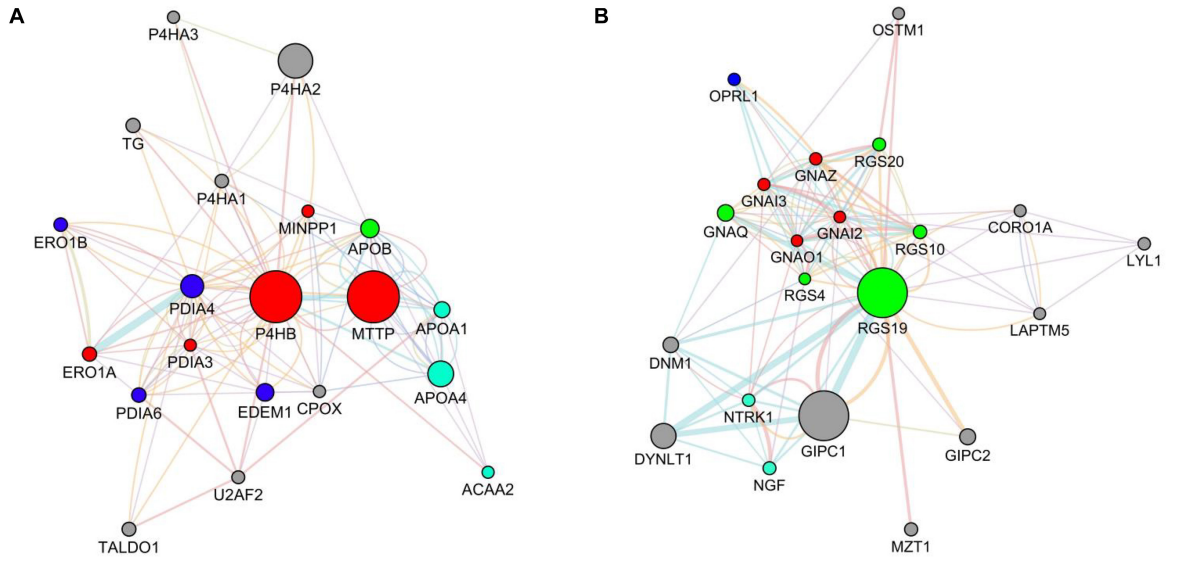
Independence of the Prognostic Value of P4HB and RGS19 From Clinical Variables

To clarify whether P4HB and RGS19 were prognostic factors independent of other clinical variables, we performed univariable and multivariable Cox regression analyses with P4HB, RGS19, and clinical features (age, gender, cancer stage, and pathological TNM stage) as covariates. The results of the univariable and multivariate Cox regression analysis demonstrated that age, pathological N and high P4HB mRNA expression were independent and unfavorable biomarkers of OS. However, RGS19 mRNA expression had no significant relationship with OS in the univariable and multivariate Cox regression analyses (**Figures 9C,D**). The results of the ROC curve analysis showed that an AUC of 0.624 was achieved for P4HB, suggesting that P4HB may be an independent unfavorable prognostic biomarker of BUC (**Figure 9E**). The prognostic value of P4HB was further validated in the independent set (GSE32548) and combined sets (TCGA, GSE13507, GSE3164, GSE32548, and GSE48075) using the OSblca database[18] (24). Consistent with the results of the TCGA dataset, P4HB was able to serve as a predictive factor for the prognosis of BUC (**Figures 9F,G**).

Experimental Verification of P4HB in BUC Cell Lines

Prolyl 4-hydroxylase, beta polypeptide expression patterns were further validated in 4 BUC cell lines (T24, J82, EJ, and SW780) and normal human uroepithelial cells (SV-HUC-1) using qRT-PCR and western blot analyses. Compared with the expression levels in SV-HUC-1 uroepithelial cells, the mRNA and protein expression levels of P4HB in the 4 BUC cell lines (T24, J82, EJ,

[18]http://bioinfo.henu.edu.cn/



P4HB

GO id	Annotation	q-value
GO:0005788	endoplasmic reticulum lumen	2.30E-10
GO:0006457	protein folding	3.64E-07
GO:0017127	cholesterol transporter activity	1.33E-06
GO:0042157	lipoprotein metabolic process	1.50E-06

RGS19

GO id	Annotation	q-value
GO:0019897	extrinsic component of plasma membrane	2.70E-07
GO:0007187	G-protein coupled receptor signaling pathway	8.42E-06
GO:0005096	GTPase activator activity	2.06E-04
GO:0007202	activation of phospholipase C activity	2.63E-02

FIGURE 6 | Identification of the biological functions of P4HB and RGS19 using the GeneMANIA database. Biological function analyses revealed the gene set that was enriched in the target network of P4HB **(A)** and RGS19 **(B)**. The different colors for the network nodes indicate the biological functions of the enriched set.

and SW480) were significantly increased by (8.34 ± 0.75)-fold and (5.20 ± 0.23)-fold, (5.52 ± 1.10)-fold and (3.55 ± 0.35)-fold, (11.30 ± 0.94)-fold and (4.31 ± 0.44)-fold, and (6.47 ± 0.82)-fold and (2.5 ± 0.22)-fold ($P < 0.05$), respectively (**Figure 10A**). Since the expression level of P4HB was upregulated in bladder cancer tissues and cells, we further investigated the effect of silencing P4HB on the viability and invasion of BUC cells *in vitro*. As expected, transfection with P4HB-specific siRNA in T24 and EJ cells dramatically inhibited cell invasion and proliferation, consistent with the decreased expression levels of P4HB (**Figures 10B–D**), compared with the control group. The viability of T24 cells and EJ cells at 12, 24, 36, 48, and 72 h in the P4HB-specific siRNA group was (91.8 ± 3.2)% and (89.2 ± 3.0)%, (83.3 ± 5.4)% and (78.5 ± 5.0)%, (64.8 ± 7.6)% and (70.4 ± 7.2)%, (40.2 ± 8.1)% and (55.6 ± 8.3)%, and (37.5 ± 4.2)% and (52.0 ± 3.8)%, respectively (**Figure 10C**). The results of invasion assays demonstrated that the invasion of T24 cells and EJ cells was significantly decreased by (0.35 ± 0.12)-fold and (0.42 ± 0.14)-fold ($P < 0.05$), respectively (**Figure 10D**). These results further supported that regulation of P4HB may be responsible for the development of BUC.

DISCUSSION

Autophagy is a multistep, multistage and multifactorial complex biological process. Numerous ARGs and signaling pathways have been reported to be involved in the regulation of autophagy (25–27). Increasing evidence has shown that autophagy is an important mechanism of tumorigenesis and that interfering with autophagy signaling by targeting key ARGs may be a novel therapeutic strategy for cancer treatment. Previously, researchers have confirmed that four ARGs, including hypoxia inducible factor-1 (HIF-1α), autophagy-related 7 (ATG7), sestrin 2 (SENS2), and beclin 1 (BECN1), were associated with the cell proliferation, apoptosis and invasion of BUC cells (28–31). However, the potential clinical value of ARGs for the prognosis of patients with BUC remains unclarified.

In the present study, we first obtained 232 ARGs from the HADb and further identified 37 differentially expressed ARGs in the onset of BUC based on the TCGA database. Subsequently, a functional enrichment analysis demonstrated that these aberrantly expressed ARGs influenced apoptosis, ER stress, the cell cycle and several cancer-related pathways. We then analyzed the correlation between differentially expressed ARGs and the prognosis of patients with BUC. The results revealed that BUC patients with high expression of P4HB and RGS19 have poor OS and DFS, respectively. Thus, we next focused on investigating the biological roles of P4HB and RGS19 in BUC. The overexpression of P4HB and RGS19 was further validated in BUC using independent data sets, including those from the Oncomine and GEO databases. Moreover, cBioPortal analysis indicated that P4HB and RGS19

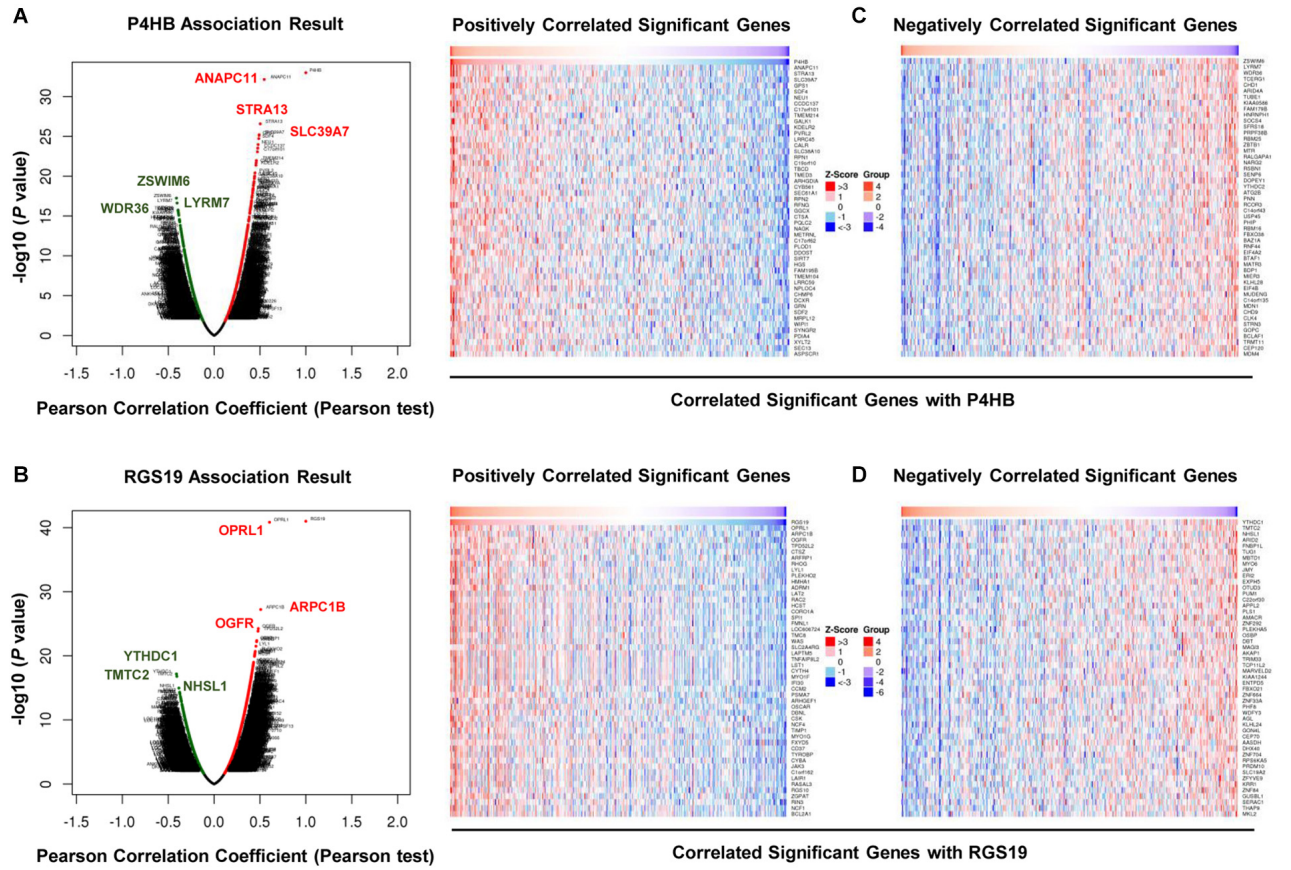


FIGURE 7 | Differentially expressed gene correlations with P4HB or RGS19 in BUC (LinkedOmics). A Pearson test was used to analyze the correlation between P4HB **(A)** or RGS19 **(B)** and differentially expressed genes in BUC. Heat maps showing genes positively and negatively correlated with P4HB **(C)** or RGS19 **(D)** in BUC (top 50 genes). Red indicates positively correlated genes, and green indicates negatively correlated genes.

mRNA expression was significantly associated with mutations and alterations. Given that P4HB and RGS19 are the potential clinical values of ARGs for BUC, we further investigated the clinical significance of P4HB and RGS19 and found that these two genes were closely related to nodal metastasis and cancer stage.

Prolyl 4-hydroxylase, beta polypeptide, also known as protein disulfide-isomerase family A member 1 (PDIA1), is the main member of the PDI gene family and is identified primarily as a multifunctional protein involved in ER stress and the unfolded protein response (UPR) (32). An accumulation of UPRs in the ER leads to stress conditions and induces an autophagic response (33). Several studies have linked P4HB to various human cancers, including brain, colon, kidney and gastric cancer (34–37). Sun et al. found that P4HB could attenuate temozolomide resistance in malignant glioma via the ER stress response pathway (34). Xie et al. reported that P4HB was associated with tumor progression and the therapeutic outcome of kidney renal clear cell carcinoma (36). RGS19 is a prototypical GTPase-activating protein with multiple functions. Recent studies suggest that RGS19 can regulate autophagy by directly detecting extracellular nutrients (38, 39). Emerging studies show that RGS19 also modulates cell proliferation by forming signaling complexes with growth factor receptors. Overexpression of RGS19 could induce increased cell proliferation via enhanced Akt signaling and the deregulation of cell cycle control (40). Wang et al. reported that RGS19 suppressed Ras-induced neoplastic transformation and tumorigenesis of non-small-cell carcinoma (41). However, few reports have been published regarding the effects and mechanisms of action of P4HB and RGS19 in BUC. Therefore, we further investigated the potential functions of P4HB and RGS19 in BUC using co-expression network analysis and GSEA. We found that P4HB and RGS19 may influence the progression and prognosis of BUC by regulating the ER stress response, cytokine-mediated signaling pathway and inflammatory response. The development and progression of bladder cancer involves multiple factors, such as clinicopathological features. We further analyzed whether P4HB and RGS19 are independent poor prognosis factors in BUC using multivariate Cox proportional hazards regression analysis. The results revealed that P4HB but not RGS19

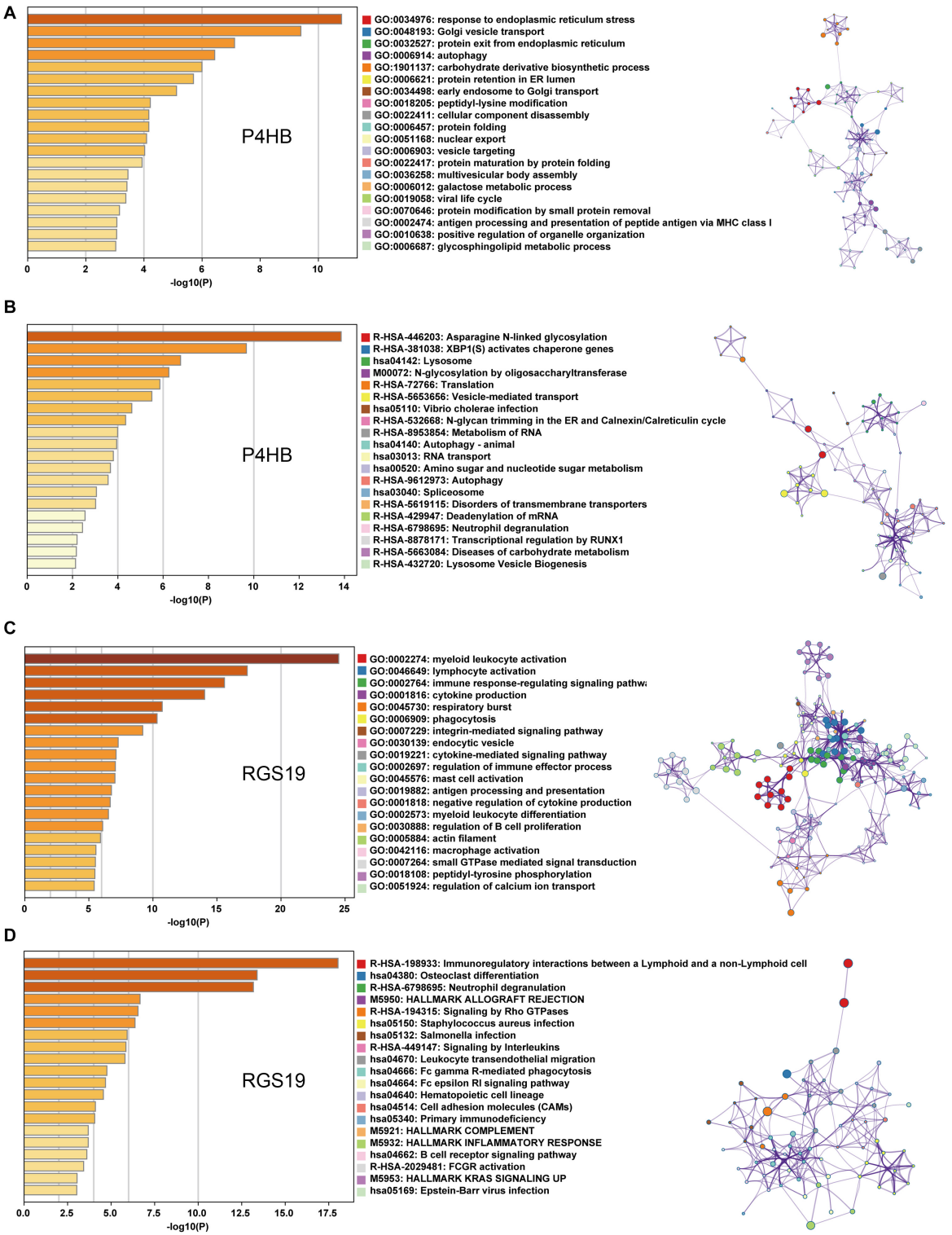


FIGURE 8 | GO, KEGG and Reactome analyses of the mRNA targets of P4HB and RGS19. **(A,B)** Enrichment analyses based on GO and KEGG/Reactome pathways to predict the potential function of mRNAs targeted by P4HB. **(C,D)** Enrichment analyses based on GO and KEGG/Reactome pathways to predict the potential functions of mRNAs targeted by RGS19. The relationships among the enriched clusters from the GO and KEGG/Reactome analyses were visualized with Metascape.

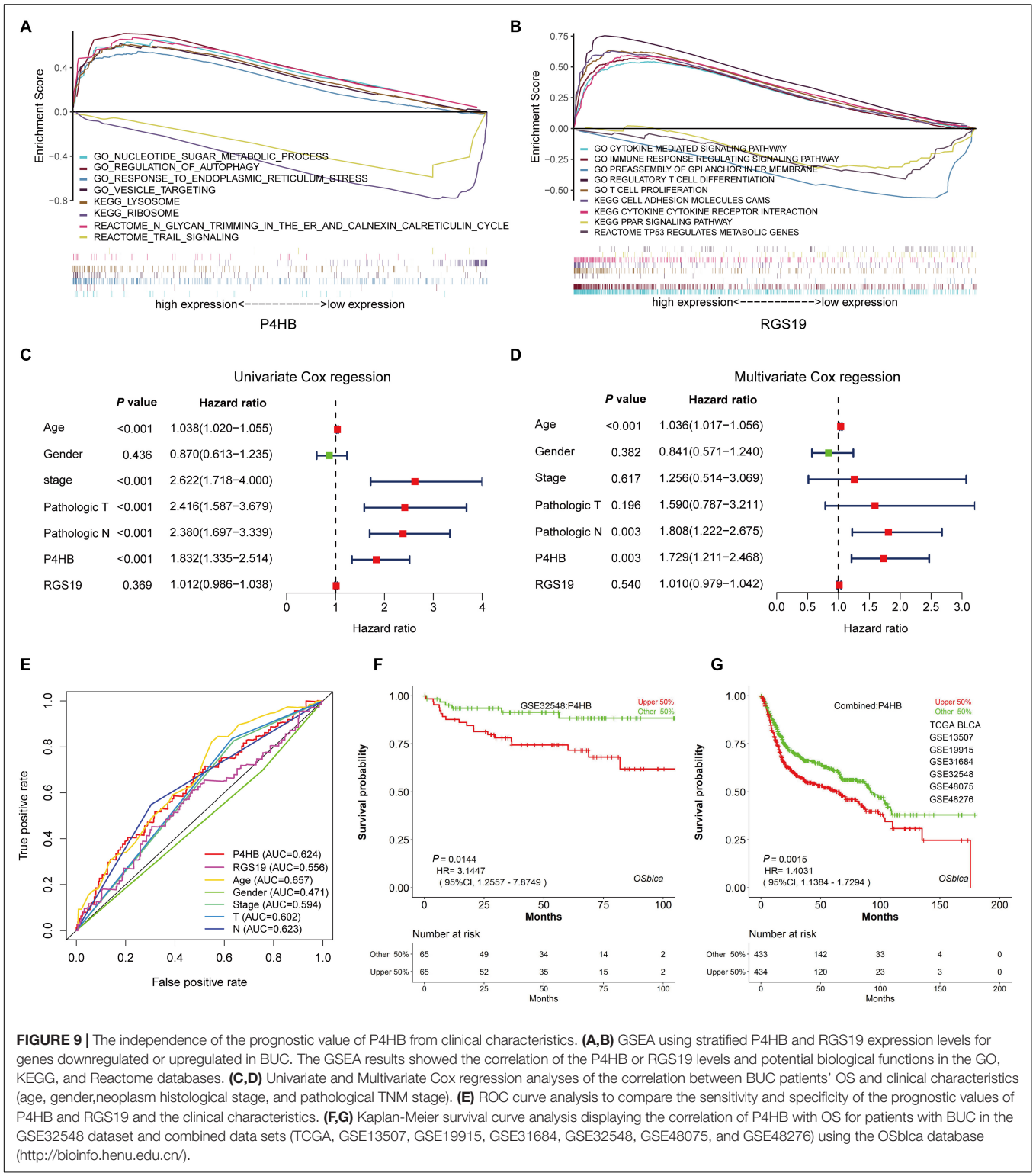


FIGURE 9 | The independence of the prognostic value of P4HB from clinical characteristics. **(A,B)** GSEA using stratified P4HB and RGS19 expression levels for genes downregulated or upregulated in BUC. The GSEA results showed the correlation of the P4HB or RGS19 levels and potential biological functions in the GO, KEGG, and Reactome databases. **(C,D)** Univariate and Multivariate Cox regression analyses of the correlation between BUC patients' OS and clinical characteristics (age, gender,neoplasm histological stage, and pathological TNM stage). **(E)** ROC curve analysis to compare the sensitivity and specificity of the prognostic values of P4HB and RGS19 and the clinical characteristics. **(F,G)** Kaplan-Meier survival curve analysis displaying the correlation of P4HB with OS for patients with BUC in the GSE32548 dataset and combined data sets (TCGA, GSE13507, GSE19915, GSE31684, GSE32548, GSE48075, and GSE48276) using the OSblca database (http://bioinfo.henu.edu.cn/).

is an independent unfavorable biomarker from conventional clinical factors (age, gender, cancer stage, and pathological TNM stage), indicating that P4HB may potentially enable clinicians to discriminate high-risk patients from low-risk patients with identical clinical characteristics for rationalizing treatment decisions. We investigated the biological effect of P4HB in BUC cells *in vitro*. We found that the mRNA and protein expression of P4HB in BUC cells was upregulated compared with the expression in SV-HUC-1 uroepithelial cells, and knockdown of P4HB dramatically inhibited the cell

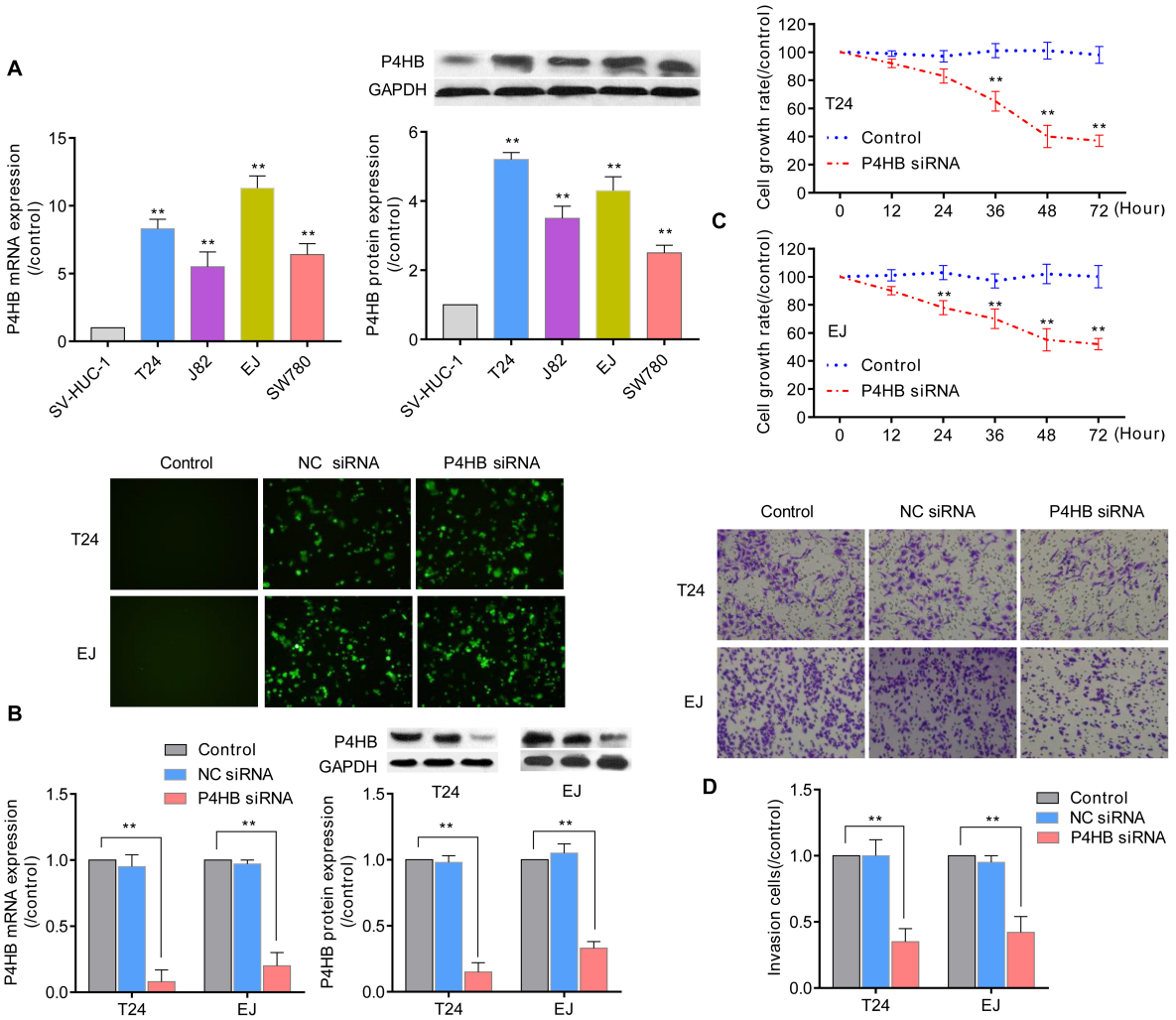


FIGURE 10 | The effect of P4HB on cell invasion and viability of BUC cells. **(A)** Detection of the P4HB expression level using qRT-PCR and western blot analyses in 4 BUC cell lines (T24, J82, EJ, and SW780) and SV-HUC-1 uroepithelial cells. **(B)** After transfection with P4HB-specific siRNA for 24 h in T24 and EJ cells, the mRNA and protein expression levels of P4HB were determined by qRT-PCR and western blot analyses, respectively. Representative images of transfection are shown at 100 × magnification. **(C)** T24 and EJ cells were transfected with P4HB-specific siRNA for the indicated periods, and the cell viabilities were determined by an MTT assay. **(D)** T24 and EJ cells were transfected with P4HB-specific siRNA for 24 h and incubated for an additional 24 h in the Boyden chemotaxis chamber, and the cell invasive ability was investigated by invasion assays. Data are representative images or expressed as the mean ± SD of each group of cells from three independent experiments, $^{**}P < 0.01$ vs. the SV-HUV-1 or control group.

invasion and proliferation of BUC cells. These preliminary *in vitro* experimental results confirmed that P4HB may be associated with the development and progression of bladder cancer. Previous studies have demonstrated that PH4B can induce malignant tumor cell proliferation, invasion and metastasis by regulating hypoxia inducible factor-1α (HIF-1α) expression and the MAPK signaling pathway (42, 43). In our present study, we found that P4HB expression was positively associated with the expression levels of ANAPC11, STRA13, and SLC397A and negatively associated with the expression levels of ZSWIM6, LYRM7, and WDR36 (**Figure 7A**). However, few reports have demonstrated that these genes, which are closely related to PH4B, are associated with the development of BUC. Interestingly, we performed GSEA to predict the potential biological functions of P4HB expression-related genes and found that these genes were involved in the nucleotide sugar metabolic process, regulation of autophagy, the response to ER stress and vesicle targeting (**Figure 9A**), suggesting that P4HB may provide some new insights into the molecular mechanism of BUC and novel treatment targets.

CONCLUSION

In this study, we identified P4HB from hundreds of candidate ARGs in large-scale BUC samples; P4HB can be used as a diagnostic and prognostic marker for patients with BUC and can

also provide a better understanding of the regulatory mechanisms of autophagy involved in the development of BUC. Moreover, there are still some limitations of the present study that should be acknowledged. We will perform further experimental research *in vitro* and *in vivo* to investigate the precise functions and mechanisms of P4HB in the regulation of autophagy-mediated tumorigenesis in BUC.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

AUTHOR CONTRIBUTIONS

LL and CZ designed the experiments. LL, WX, and FZ performed the experiments. LL and WX drafted the manuscript. LL, TH, YF, and JY conducted the data analysis and interpreted the results. LL and CZ revised the manuscript. All authors reviewed and approved the manuscript.

FUNDING

This study was supported by grants from the National Natural Science Foundation of China (81502204), the Hubei Province Nature Science Foundation of China (2014CFB399 and 2017CFB24), and Wuhan Clinical Medical Research of China (WX14A01).

REFERENCES

1. Zhou M, Wang H, Zeng X, Yin P, Zhu J, Chen W, et al. Mortality, morbidity, and risk factors in China and its provinces, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet*. (2019) 394:1145–58. doi: 10.1016/S0140-6736(19)30427-1
2. Massari F, Santoni M, Ciccarese C, Brunelli M, Conti A, Santini D, et al. Emerging concepts on drug resistance in bladder cancer: implications for future strategies. *Crit Rev Oncol Hematol*. (2015) 96:81–90. doi: 10.1016/j.critrevonc.2015.05.005
3. Nadal R, Bellmunt J. Management of metastatic bladder cancer. *Cancer Treat Rev*. (2019) 76:10–21. doi: 10.1016/j.ctrv.2019.04.002
4. Tan WS, Tan WP, Tan MY, Khetrapal P, Dong L, deWinter P, et al. Novel urinary biomarkers for the detection of bladder cancer: a systematic review. *Cancer Treat Rev*. (2018) 69:39–52. doi: 10.1016/j.ctrv.2018.05.012
5. Chang IW, Wu WJ, Wang YH, Wu TF, Liang PI, He HL, et al. BCAT1 overexpression is an indicator of poor prognosis in patients with urothelial carcinomas of the upper urinary tract and urinary bladder. *Histopathology*. (2016) 68:520–32. doi: 10.1111/his.12778
6. Zhang Q, Wang HJ, Zhang DH, Ru GQ, He XJ, Ma YY. High expression of HEF1 is associated with poor prognosis in urinary bladder carcinoma. *OncoTargets Ther*. (2014) 7:1319–26. doi: 10.2147/OTT.S64418
7. Mowers EE, Sharifi MN, Macleod KF. Functions of autophagy in the tumor microenvironment and cancer metastasis. *FEBS J*. (2018) 285:1751–66. doi: 10.1111/febs.14388
8. Levy JMM, Towers CG, Thorburn A. Targeting autophagy in cancer. *Nat Rev Cancer*. (2017) 17:528–42. doi: 10.1038/nrc.2017.53
9. Kocaturk NM, Akkoc Y, Kig C, Bayraktar O, Gozuacik D, Kutlu O. Autophagy as a molecular target for cancer treatment. *Eur J Pharm Sci*. (2019) 134:116–37. doi: 10.1016/j.ejps.2019.04.011
10. Folkerts H, Hilgendorf S, Vellenga E, Bremer E, Wiersma VR. The multifaceted role of autophagy in cancer and the microenvironment. *Med Res Rev*. (2019) 39:517–60. doi: 10.1002/med.21531
11. Li YJ, Lei YH, Yao N, Wang CR, Hu N, Ye WC, et al. Autophagy and multidrug resistance in cancer. *Chin J Cancer*. (2017) 36:52. doi: 10.1186/s40880-017-0219-2
12. White E, Mehnert JM, Chan CS. Autophagy, metabolism, and Cancer. *Clin Cancer Res*. (2015) 21:5037–46. doi: 10.1158/1078-0432.CCR-15-0490
13. Zeng Q, Liu J, Cao P, Li J, Liu X, Fan X, et al. Inhibition of REDD1 sensitizes bladder urothelial carcinoma to paclitaxel by inhibiting autophagy. *Clin Cancer Res*. (2018) 24:445–59. doi: 10.1158/1078-0432.CCR-17-0419
14. Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nat Commun*. (2019) 10:1523. doi: 10.1038/s41467-019-09234-6
15. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA*. (2005) 102:15545–50. doi: 10.1073/pnas.0506580102
16. Tang Z, Kang B, Li C, Chen T, Zhang Z. GEPIA2: an enhanced web server for large-scale expression profiling and interactive analysis. *Nucleic Acids Res*. (2019) 47:W556–60. doi: 10.1093/nar/gkz430
17. Modlich O, Prisack HB, Pitschke G, Ramp U, Ackermann R, Bojar H, et al. Identifying superficial, muscle-invasive, and metastasizing transitional cell carcinoma of the bladder: use of cDNA array analysis of gene expression profiles. *Clin Cancer Res*. (2004) 10:3410–21. doi: 10.1158/1078-0432.CCR-03-0134
18. Sanchez-Carbayo M, Socci ND, Lozano J, Saint F, Cordon-Cardo C. Defining molecular profiles of poor outcome in patients with invasive bladder cancer using oligonucleotide microarrays. *J Clin Oncol*. (2006) 24:778–89. doi: 10.1200/JCO.2005.03.2375
19. Dyrskjot L, Kruhoffer M, Thykjaer T, Marcussen N, Jensen JL, Moller K, et al. Gene expression in the urinary bladder: a common carcinoma in situ gene expression signature exists disregarding histopathological classification. *Cancer Res*. (2004) 64:4040–8. doi: 10.1158/0008-5472.CAN-03-3620
20. Lee JS, Leem SH, Lee SY, Kim SC, Park ES, Kim SB, et al. Expression signature of E2F1 and its associated genes predict superficial to invasive progression of bladder tumors. *J Clin Oncol*. (2010) 28:2660–7. doi: 10.1200/JCO.2009.25.0977
21. Chandrashekar DS, Bashel B, Balasubramanya SAH, Creighton CJ, Ponce-Rodriguez I, Chakravarthi B, et al. UALCAN: a portal for facilitating tumor subgroup gene expression and survival analyses. *Neoplasia*. (2017) 19:649–58. doi: 10.1016/j.neo.2017.05.002
22. Vasaikar SV, Straub P, Wang J, Zhang B. LinkedOmics: analyzing multi-omics data within and across 32 cancer types. *Nucleic Acids Res*. (2018) 46:D956–63. doi: 10.1093/nar/gkx1090
23. Warde-Farley D, Donaldson SL, Comes O, Zuberi K, Badrawi R, Chao P, et al. The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. *Nucleic Acids Res*. (2010) 38:W214–20. doi: 10.1093/nar/gkq537
24. Zhang GS, Wang Q, Yang MS, Yuan Q, Dang YF, Sun XX, et al. OSblca: a web server for investigating prognostic biomarkers of bladder cancer patients. *Front Oncol*. (2019) 9:466. doi: 10.3389/fonc.2019.00466
25. Ye X, Zhou XJ, Zhang H. Exploring the role of autophagy-related gene 5 (ATG5) yields important insights into autophagy in autoimmune/autoinflammatory diseases. *Front Immunol*. (2018) 9:2334. doi: 10.3389/fimmu.2018.02334
26. Kim SY, Yang CS, Lee HM, Kim JK, Kim YS, Kim YR, et al. ESRRA (estrogen-related receptor alpha) is a key coordinator of transcriptional and post-translational activation of autophagy to promote innate host defense. *Autophagy*. (2018) 14:152–68. doi: 10.1080/15548627.2017.1339001
27. Yu L, Chen Y, Tooze SA. Autophagy pathway: cellular and molecular mechanisms. *Autophagy*. (2018) 14:207–15. doi: 10.1080/15548627.2017.1378838

28. Yang X, Yin H, Zhang Y, Li X, Tong H, Zeng Y, et al. Hypoxia-induced autophagy promotes gemcitabine resistance in human bladder cancer cells through hypoxia-inducible factor 1alpha activation. *Int J Oncol*. (2018) 53:215–24. doi: 10.3892/ijo.2018.4376
29. Zhu J, Tian Z, Li Y, Hua X, Zhang D, Li J, et al. ATG7 promotes bladder cancer invasion via autophagy-mediated increased ARHGDIB mRNA stability. *Adv Sci*. (2019) 6:1801927. doi: 10.1002/advs.201801927
30. Hua X, Xu J, Deng X, Xu J, Li J, Zhu DQ, et al. New compound ChlA-F induces autophagy-dependent anti-cancer effect via upregulating Sestrin-2 in human bladder cancer. *Cancer Lett*. (2018) 436:38–51. doi: 10.1016/j.canlet.2018.08.013
31. Lin JF, Lin YC, Tsai TF, Chen HE, Chou KY, Hwang TI. Cisplatin induces protective autophagy through activation of BECN1 in human bladder cancer cells. *Drug Des Devel Ther*. (2017) 11:1517–33. doi: 10.2147/DDDT.S126464
32. Perri E, Parakh S, Atkin J. Protein disulphide isomerases: emerging roles of PDI and ERp57 in the nervous system and as therapeutic targets for ALS. *Expert Opin Ther Targets*. (2017) 21:37–49. doi: 10.1080/14728222.2016.1254197
33. Luo B, Lee AS. The critical roles of endoplasmic reticulum chaperones and unfolded protein response in tumorigenesis and anticancer therapies. *Oncogene*. (2013) 32:805–18. doi: 10.1038/onc.2012.130
34. Sun S, Lee D, Ho AS, Pu JK, Zhang XQ, Lee NP, et al. Inhibition of prolyl 4-hydroxylase, beta polypeptide (P4HB) attenuates temozolomide resistance in malignant glioma via the endoplasmic reticulum stress response (ERSR) pathways. *Neuro Oncol*. (2013) 15:562–77. doi: 10.1093/neuonc/not005
35. Zhou Y, Yang J, Zhang Q, Xu Q, Lu L, Wang J, et al. P4HB knockdown induces human HT29 colon cancer cell apoptosis through the generation of reactive oxygen species and inactivation of STAT3 signaling. *Mol Med Rep*. (2019) 19:231–7. doi: 10.3892/mmr.2018.9660
36. Xie L, Li H, Zhang L, Ma X, Dang Y, Guo J, et al. Autophagy-related gene P4HB: a novel diagnosis and prognosis marker for kidney renal clear cell carcinoma. *Aging*. (2020) 12:1828–42. doi: 10.18632/aging.102715
37. Zhang J, Wu Y, Lin YH, Guo S, Ning PF, Zheng ZC, et al. Prognostic value of hypoxia-inducible factor-1 alpha and prolyl 4-hydroxylase beta polypeptide overexpression in gastric cancer. *World J Gastroenterol*. (2018) 24:2381–91. doi: 10.3748/wjg.v24.i22.2381
38. Ogier-Denis E, Petiot A, Bauvy C, Codogno P. Control of the expression and activity of the Galpha-interacting protein (GAIP) in human intestinal cells. *J Biol Chem*. (1997) 272:24599–603. doi: 10.1074/jbc.272.39.24599
39. Wu T, Li Y, Huang D, Han F, Zhang YY, Zhang DW, et al. Regulator of G-protein signaling 19 (RGS19) and its partner Galpha-inhibiting activity polypeptide 3 (GNAI3) are required for zVAD-induced autophagy and cell death in L929 cells. *PLoS One*. (2014) 9:e94634. doi: 10.1371/journal.pone.0094634
40. Tso PH, Yung LY, Wang Y, Wong YH. RGS19 stimulates cell proliferation by deregulating cell cycle control and enhancing Akt signaling. *Cancer Lett*. (2011) 309:199–208. doi: 10.1016/j.canlet.2011.06.002
41. Wang Y, Tong Y, Tso PH, Wong YH. Regulator of G protein signaling 19 suppresses Ras-induced neoplastic transformation and tumorigenesis. *Cancer Lett*. (2013) 339:33–41. doi: 10.1016/j.canlet.2013.07.025
42. Zhang J, Guo S, Wu Y, Zheng ZC, Wang Y, Zhao Y. P4HB, a novel hypoxia target gene related to gastric cancer invasion and metastasis. *Biomed Res Int*. (2019) 2019:9749751. doi: 10.1155/2019/9749751
43. Sun S, Kiang KMY, Ho ASW, Lee D, Poon MW, Xu FF, et al. Endoplasmic reticulum chaperone prolyl 4-hydroxylase, beta polypeptide (P4HB) promotes malignant phenotypes in glioma via MAPK signaling. *Oncotarget*. (2017) 8:71911–23. doi: 10.18632/oncotarget.18026

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Lyu, Xiang, Zheng, Huang, Feng, Yuan and Zhang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Oncology

ORIGINAL RESEARCH
published: 19 August 2020
doi: 10.3389/fonc.2020.01411

Comparison of Stage 4 and Stage 4s Neuroblastoma Identifies Autophagy-Related Gene and LncRNA Signatures Associated With Prognosis

*Xinyao Meng†, Honglin Li†, Erhu Fang, Jiexiong Feng and Xiang Zhao**

Department of Pediatric Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

OPEN ACCESS

Edited by:
Daniel Hector Grasso, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

Reviewed by:
Xiaodan Zhong, Jilin University, China
Yulin Wang, First Affiliated Hospital of Shantou University Medical College, China

***Correspondence:**
Xiang Zhao
zhaoxiang8609@163.com

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *08 May 2020*
Accepted: *03 July 2020*
Published: *19 August 2020*

Citation:
Meng X, Li H, Fang E, Feng J and Zhao X (2020) Comparison of Stage 4 and Stage 4s Neuroblastoma Identifies Autophagy-Related Gene and LncRNA Signatures Associated With Prognosis. Front. Oncol. 10:1411. doi: 10.3389/fonc.2020.01411

Background: The spontaneous regression of neuroblastoma (NB) is most prevalent and well-documented in stage 4s NB patients. However, whether autophagy plays roles in the spontaneous regression of NB is unknown.

Objective: This study aimed to identify autophagy-related genes (ARGs) and autophagy-related long non-coding RNAs (lncRNAs) differentially expressed in stage 4 and stage 4s NB and to build prognostic risk signatures on the basis of the ARGs and autophagy-related lncRNAs.

Methods: One RNA-sequence (RNA-Seq) dataset (TARGET NBL, $n = 153$) was utilized as discovery cohort, and two microarray datasets ($n = 498$ and $n = 223$) were used as validation cohorts. Differentially expressed ARGs were identified by comparing stage 4s and stage 4 NB samples. An ARG signature risk score and an autophagy-related lncRNA signature risk score were constructed. The receiver operating characteristic (ROC) curve analyses were used to evaluate the survival prediction ability of the two signatures. Gene function annotation and Gene Set Enrichment Analysis (GSEA) were performed to clarify the autophagic biological processes enriched in different risk groups.

Results: Nine ARGs were integrated into the ARG signature. Patients in the high-risk group of the ARG signature had significantly poorer overall survival (OS) than patients in the low-risk group. The ROC curves analyses revealed that the ARG signature performed very well in predicting OS [5-year area under the curve (AUC) = 0.81]. Seven autophagy-related lncRNAs were integrated into the autophagy-related lncRNA signature. Patients in the high-risk group of the lncRNA signature had significantly poorer OS than patients in the low-risk group. The ROC curve analyses also revealed that the lncRNA signature performed well in predicting OS (5-year AUC = 0.77). Both the ARG signature and lncRNA signature are independent with other clinical risk factors in the multivariate Cox regression survival analyses. GSEAs revealed that autophagy-related biological processes are enriched in low-risk groups.

Conclusions: Autophagy-related genes and lncRNAs are differentially expressed between stage 4 and stage 4s NB. The ARG signature and autophagy-related lncRNA signature successfully stratified NB patients into two risk groups. Autophagy-related biological processes are highly enriched in low-risk NB groups.

Keywords: autophagy, neuroblastoma, stage 4s, prognosis, long non-coding RNA

INTRODUCTION

Spontaneous regression of cancer has been documented since the 1900s (1). It means that a malignant tumor completely or partially disappears without acceptance of any tumor-associated treatment (1). This interesting and promising biological phenomenon has been observed in various types of cancers (2–6). However, neuroblastoma (NB) is generally considered the most common malignancy in which this phenomenon is most evident and prevalent (3, 7). The spontaneous regression of NB has been validated by several mass screening programs undertaken in different regions of the world including Japan, North America, and Europe or (8–11). This phenomenon is most evident in NB patients with stage 4s disease (3, 12–14). Patients with stage 4s NB usually had a localized primary tumor but with tumors metastasized to the liver, skin, or bone marrow (7). Unlike other metastatic malignancies, NB patients with stage 4s disease generally had a surprisingly good survival outcome, and most of them underwent spontaneous regression even without antitumor treatment (15–17). One study reported a 5-year overall survival (OS) rate of 92% for stage 4s NB patients receiving supportive care or minimal therapy (18). One recent study also reported a complete regression rate of 92% for stage 4s adrenal NB (19).

Spontaneous regression is not restricted to stage 4s NB; it also regularly occurs in infants with localized NB (one study reported a complete regression rate of about 38.6% for localized NB) (20). In fact, it can be observed in any stage of NB if the tumor has biologically favorable histology (7, 15). Because spontaneous regression of NB is most prevalent in patients with stage 4s disease, investigators have been focusing on stage 4s NB as a surrogate to explore the underlining mechanisms responsible for spontaneous regression of NB (7, 12–14). However, the mechanism responsible for the spontaneous regression of NB is still largely unknown.

In recent years, autophagy has been found to play important roles in tumor development and progression (21, 22) and is also involved in NB (23–26). The association between autophagy and spontaneous regression of NB is unknown. Because studies have found that autophagy is associated with NB cell apoptosis and differentiation (23, 24), we wish to know whether autophagy is involved in the process of spontaneous regression.

In this study, as other investigators have done previously (7, 12–14), we also use stage 4s NB as a surrogate. One RNA-sequence (RNA-Seq) datasets (TARGET NBL, $n = 153$) and two microarray datasets ($n = 498$ and $n = 223$) were utilized in this study. Differentially expressed autophagy-related genes (ARGs) were identified by comparing those deceased cases in stage 4 NB and those survived cases in stage 4s NB. As one of our previous study has done before (7), the dead cases in stage 4s were excluded to make it better for serving as surrogates to NBs that underwent spontaneous regression.

Finally, nine differentially expressed and survival-related ARGs were incorporated into the ARG prognostic signature. Seven autophagy-related long non-coding RNAs (lncRNAs) were also identified and incorporated into an autophagy-related lncRNA prognostic signature. The ARG signature and autophagy-related lncRNA signature performed well in predicting OS of NB patients. Gene Ontology (GO) function annotation and Gene Set Enrichment Analysis (GSEA) revealed that autophagy-related biological processes were significantly enriched in the low-risk groups, whereas no autophagy gene set was identified in the high-risk groups. These results reveal that autophagy tends to play tumor-suppressive roles in NB and might be associated with the spontaneous regression of NB.

MATERIALS AND METHODS

Neuroblastoma Dataset Processing

The processed data of the RNA-Seq dataset (TARGET NBL, $n = 153$) were downloaded from National Cancer Institute GDC Data Portal. The original data of the TARGET NBL obtained from GDC Data Portal have a total of 161 samples. Two paired duplicated samples were identified; the gene express values in the duplicated sample are the same too, and thus, we excluded these duplicated samples during the analysis. We also identified that six paired samples are from the same patients. The clinical information for these paired samples is the same, whereas one sample was obtained from the original tumor and the other one sample was obtained from recurrent tumor. In order to reduce confounding factors, we also excluded those six recurrent tumor samples and kept their corresponding primary tumor samples only. Finally, 153 samples were kept for the analyses, with 73 stage 4 NB samples from patients who died and 19 stage 4s samples from patients who survived during the follow-up.

The processed data of the Agilent microarray datasets GSE49710 ($n = 498$) were obtained from Gene Expression Omnibus (GEO) database. The processed data of the Agilent microarray datasets E-MTAB-8248 ($n = 223$) were obtained from ArrayExpress database. The genes express levels in the three datasets were already processed and log2 transformed. The clinical characteristics of the patients in these three datasets were also obtained and are shown in **Supplementary Table 1**. The RNA-Seq dataset (termed as cohort 1) was used as the discovery cohort. The microarray datasets GSE49710 (termed as

cohort 2) and E-MTAB-8248 (termed as cohort 3) were used as the validation cohorts. The Agilent microarray probes IDs were firstly annotated using the platform GPL16876 (Agilent-020382 Human Custom Microarray 44k); then, the probes IDs were re-annotated by their GenBank Accession number in order to renew the annotation. Finally, in order keep consistency over the three datasets, the Ensemble ID in the three datasets was transformed into gene symbols according to GRCh38.p12, and the background of the three datasets was also intersected normalized by R package "sva." If multiple probes mapped to one same gene, the average level of the expression value will be used. The online platform of cBio Cancer Genomics Portal (cBioportal) (http://www.cbioportal.org/) was utilized to analyze the genomic alteration (mutation and copy number alteration) of the identified genes (27).

Extraction of Differentially Expressed Autophagy-Related Genes

ARGs were extracted from Human Autophagy Database (https://www.autophagy.lu/), with a total of 232 ARGs. Differential expression analyses were performed by "limma" package using the R (version 3.6.2) software in cohort 1. Genes with false discovery rate (FDR) < 0.05 and $|\log2FoldChange| > 0.5$ were extracted as differentially expressed genes. LncRNAs correlated (Pearson correlation threshold ≥ 0.5) with ARGs were extracted as autophagy-related lncRNAs. Only those lncRNAs matching the GENCODE annotation of lncRNA (release 31, GRCh38.p12) were selected.

Construction of the Autophagy-Related Prognostic Signatures

Univariate Cox proportional hazards regression analyses were performed to identify those ARGs associated with OS in the entire cohort 1. A $p \leq 0.05$ was considered statistically significant. Those survival-related ARGs were put into the Cox proportional hazards model survival analysis with least absolute shrinkage and selection operator (LASSO) penalty to eliminate false positives owing to over-fitting (28). Finally, the autophagy-related prognostic signature was constructed by weighting the Cox regression coefficients for each gene to calculate a risk score for every patient. The median value was used as the cutoff value, and the patients were classified as low risk and high risk accordingly. The same formula was applied to the validation cohorts, and the same cutoff value was used to divide the patients into two risk groups. Autophagy-related lncRNA signature was constructed by the same method. Those autophagy-related lncRNAs associated with OS were put into LASSO Cox model regression analyses. The autophagy-related lncRNA signature was constructed by the same method.

Function Annotation and Gene Set Enrichment Analysis

Differentially expressed ARGs that associated with OS were put into GO functional annotation. The GO function annotation was first performed by R software using "BiocManager" package of "clusterProfiler," "org.Hs.eg.db," and "enrichplot." Then the circle plot of GO function annotation was generated by R software using package "GOplot." Functional annotation with a $p < 0.05$ was considered statistically significant. GSEA comparing low-risk group and high-risk group was performed by GSEA software (version 4.0.03). An FDR q-value < 0.25 and a nominal $p < 0.05$ were considered statistically significant for GSEAs.

Statistical Analysis

The univariate and multivariate Cox survival regression analyses were calculated by the R package "survival." The LASSO Cox survival analyses were performed by the R package "glmnet," and 1,000-fold cross-validation was used. The Kaplan–Meier survival curves were constructed by R software or GraphPad Prism 5, and the statistical significance was estimated by the two-sided log-rank test. The time-dependent receiver operating characteristic (ROC) curves and area under the curve (AUC) analyses were performed to evaluate the predictive performance of the prognostic signatures and performed by the R package "time ROC." Nomograms were generated by R package "rms," and Harrell's concordance index (C-index) was calculated to evaluate the discriminatory ability. Volcano plot was plotted by the R package "ggplot2." Heat maps were generated by the R package "pheatmap." The Pearson correlation matrix was generated by the R package "corrplot." The alluvial diagrams were generated by the R package "ggalluvial." The R software version 3.6.2 was utilized in this study for the statistical analyses. A $p < 0.05$ was considered statistically significant, and all statistical tests were two-sided.

RESULTS

Identification of Differentially Expressed and Survival-Related Autophagy-Related Genes

Differential expression analyses were performed on the RNA-Seq datasets (cohort 1, $n = 153$). Cohort 1 contains 125 stage 4 NB samples and 21 stage 4s NB samples. A total of 48 ARGs were found to be differentially expressed between those stage 4 cases who died during follow-up ($n = 73$) and those stage 4s cases who survived during follow-up ($n = 19$). Thirty-two ARGs were up-regulated in stage 4 NB samples, whereas 16 ARGs were up-regulated in stage 4s NB samples (**Figures 1A,B**).

Univariate Cox proportional model survival analyses revealed that 19 ARGs were significantly ($p < 0.05$) associated with OS in the entire cohort 1 (**Supplementary Figure 1A**). Twelve ARGs were up-regulated in stage 4s NB samples and associated with good survival, whereas seven ARGs were up-regulated in stage 4 NB samples and associated with bad survival.

Construction and Validation of Autophagy-Related Gene Prognostic Signature

The survival-related ARGs were put into LASSO Cox survival analysis to eliminate false positives (**Supplementary Figures 1B,C**). The 1 – SE criterion revealed only one gene (TM9SF1) in the model; thus, the "lambda.min"

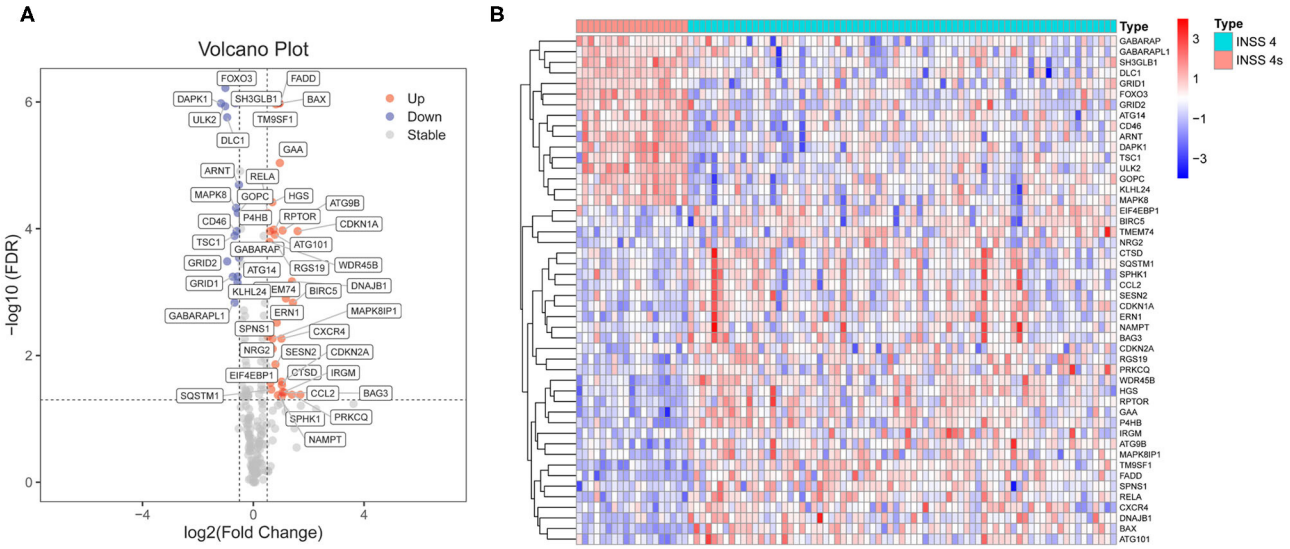


FIGURE 1 | Identification of differentially expressed autophagy-related genes (ARGs) between stage 4s and stage 4 neuroblastoma (NB). **(A)** Volcano plot shows the differentially expressed 48 ARGs in cohort 1. **(B)** The heat map shows the expression values of the identified 48 ARGs in cohort 1.

criterion was used to select the minimum lambda value (λ = 0.0677). Finally, nine ARGs (**Supplementary Table 2**) were selected and incorporated into the ARG signature risk score. The risk scores were calculated for each patient as follows: risk score = 0.1248 * SPNS1 + 0.6746 * TM9SF1 + 0.0145 * WDR45B + 0.0088 * EIF4EBP1 – 0.0012 * GABARAPL1 – 0.0649 * ATG14 – 0.0810 * ULK2 – 0.0165 * DLC1 – 0.0269 * ARNT. The median value was used as the cutoff value, and the entire cohort 1 was classified into two risk groups accordingly. The risk distribution, survival status, and gene expression pattern are shown in **Figure 2A**. The scatter plot (**Figure 2A**) shows that most of the patients in the high-risk group died and most of the patients in the low-risk group survived during the 15-year follow-up. The heat map (**Figure 2A**) shows that five ARGs were highly expressed in the low-risk group, whereas four ARGs were highly expressed in the high-risk group. Kaplan–Meier plots show that patients in the high-risk group have a significantly poorer OS than those in the low-risk group (**Figure 2B**). The 3-, 5-, and 10-year OS rates for the patients in high-risk group were 50.42, 28.97, and 16.01%, respectively, whereas the 3-, 5-, and 10-year OS rates for patients in low-risk group were 82.38, 75.28, and 70.66%, respectively. Time-dependent ROC curves reveal that the ARG signature has good performance in predicting OS in cohort 1, whereas the AUC at 3-, 5-, and 10-years were 0.75, 0.81, and 0.71, respectively (**Figure 2C**).

To corroborate the prognostic significance, the ARG signature was tested in cohort 2 (n = 498) and cohort 3 (n = 223) for validation using the same risk score formula. According to the same cut-off value as cohort 1, the validation cohorts were divided into two risk groups. The risk distribution, survival status, and gene expression pattern for cohort 2 are shown in **Figure 2D**. Kaplan–Meier plots show that patients in the high-risk group have a significantly poorer OS than those in the low-risk group in cohort 2 (**Figure 2E**). Time-dependent ROC curves reveal that the ARG signature has good performance in predicting OS in cohort 2, whereas the AUC at 3-, 5-, and 10-years was 0.72, 0.76, and 0.78, respectively (**Figure 2F**). Consistent with cohort 1 and cohort 2, the validation in cohort 3 shows similar results (**Supplementary Figure 2**).

Survival Analysis for the Autophagy-Related Gene Prognostic Signature

The univariate Cox regression survival analyses for the ARG signature risk score and other clinical risk factors in the entire cohort 1 are shown in **Figure 3A**. The ARG signature risk score is significantly associated with OS [hazard ration (HR) = 5.068; 95%CI: 3.047–8.430; $p < 0.001$] in the univariate survival analysis. Multivariate Cox survival analyses including gender (female vs. male), age status (<18 vs. ≥18 months), International Neuroblastoma Staging System (INSS) stage (INSS 2/3/4S vs. INSS 4), MYCN amplification (non-amplified vs. amplified), Children's Oncology Group (COG) risk status (low risk vs. high risk), ploidy (hyperploid vs. diploid), histology type (favorable vs. unfavorable), differentiation (differentiating vs. poorly differentiated), mitosis-karyorrhexis index (MKI) (low/intermediate vs. high), and pathology subtype (ganglioneuroblastoma vs. NB) as covariates were performed to evaluate the independent prognostic role of the ARG signature (**Figure 3B**). In cohort 1, only the ARG signature (HR = 4.372; 95%CI: 2.020–9.461, $p < 0.001$) and ploidy (HR = 1.897; 95%CI: 1.087–3.251; $p = 0.024$) were independently associated with OS (**Figure 3B**). The univariate and multivariate Cox regression survival analyses for the ARG signature and other clinical risk factors in cohort 2 are shown in **Figures 3C,D**. The ARG

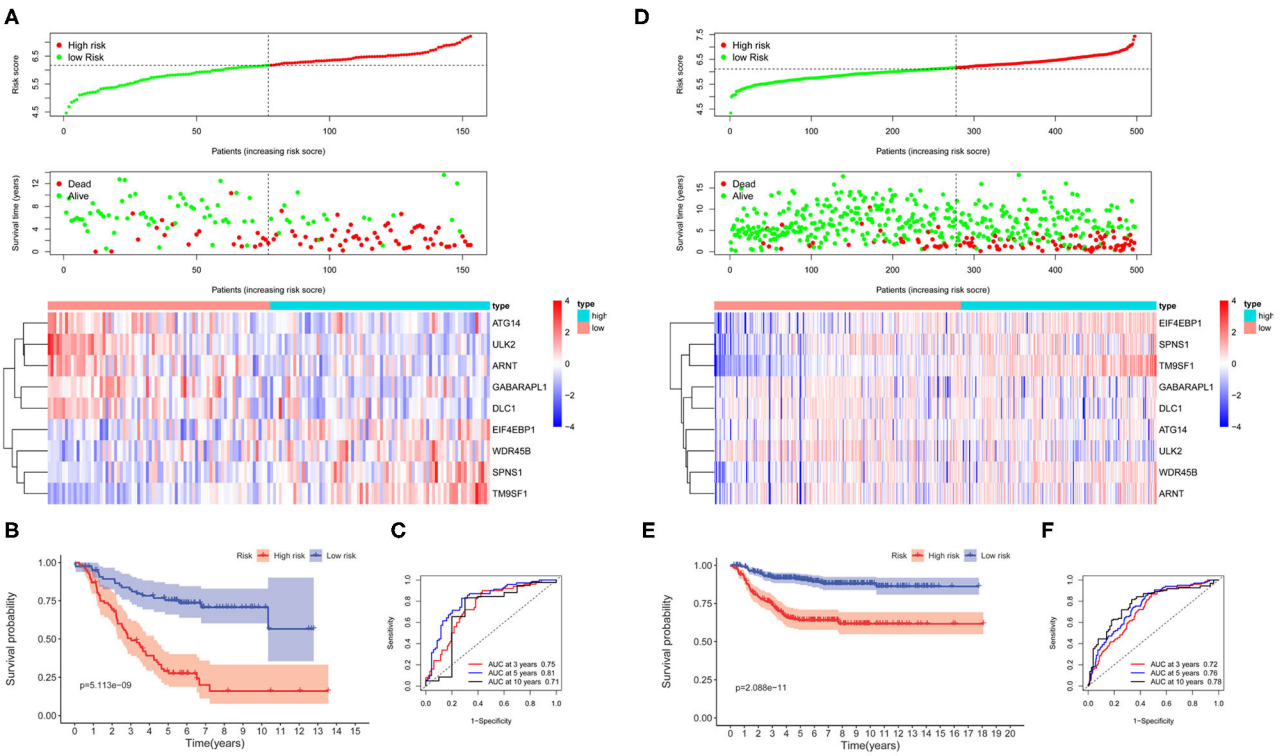


FIGURE 2 | The autophagy-related genes (ARGs) prognostic signature for neuroblastoma. **(A)** The distribution of risk scores, survival status of each patient, and heat map of ARG expression pattern in cohort 1. **(B)** Kaplan–Meier survival curve for overall survival (OS) of patients in the low-risk group and high-risk group for cohort 1. **(C)** Time-dependent receiver operating characteristic (ROC) curves for the prognostic value of the ARG signature in cohort 1. **(D)** The distribution of risk scores, survival status of each patient, and heat map of ARG expression pattern in cohort 2. **(E)** Kaplan–Meier survival curve for OS of patients in the low-risk group and high-risk group for cohort 2. **(F)** Time-dependent ROC curves for the prognostic value of the ARG signature in cohort 2.

signature risk score is significantly associated with OS in cohort 2 by both univariate model (HR = 6.077; 95%CI: 3.889–9.495; $p < 0.001$) and multivariate model (HR = 2.715; 95%CI: 1.590–4.637; $p < 0.001$). Because the COG risk group classification already considered age, MYCN amplification, and INSS stage into its risk classification system, we built a nomogram incorporating only the COG risk classification and the ARG signature risk score for prediction of OS on the basis of the largest cohort (cohort 2, $n = 498$) (**Figure 3E**). As is shown in the nomogram (**Figure 3E**), COG low risk was denoted as 0 point, whereas COG high risk was denoted as 100 points. As for the ARG signature risk score in the nomogram, a risk score of 4 was denoted as 0 point, and a risk score of 7.5 was denoted as 82 points. The risk scores between 4 and 7.5 were assigned correspondingly between 0 and 82 points and could be calculated as follows: point = (risk score – 4) * (82/3.5). The total points for the patients were calculated by combining the points for COG risk and the points for the ARG risk score, and the corresponding predicted survival probability are shown below.

The *C*-index for the nomogram was 0.84 (95%CI: 0.81–0.87), indicating a high level of accuracy. The 1-, 3-, and 5-year calibrate curves for the nomogram all revealed that the predicted OS was very close to the actual OS (**Figure 3F**). The ROC curve analyses reveal that the AUC values at 1-, 3-, and 5-years for the nomogram were higher than the AUC values at 1-, 3-, and 5-years for the COG risk, respectively (**Figure 3G**), indicating that the prognostic role of the nomogram is more accurate than the COG risk classification alone.

Prognostic Role of the Autophagy-Related Gene Signature Within Clinical Subgroups

Stratification survival analysis was performed to evaluate the prediction ability of the ARG signature in different clinical subgroups. The subgroups were classified based on MYCN amplification status, histology subtype, differentiation status, ploidy status, MKI status, pathology subtype, COG risk status, age status, and INSS stage. Within each subgroup, patients were stratified into low-risk group and high-risk group on the basis of the same cut-off value from the entire cohort 1. In the MYCN non-amplified subgroup, patients in the high-risk group had a significantly worse OS than patients in the low-risk group (**Figure 4A**), whereas the ARG signature failed to stratify patients in the MYCN amplified subgroup into two risk groups (**Figure 4B**). In both of the histology subtype (favorable and unfavorable), patients in the high-risk group had significantly worse OS than patients in the low-risk group (**Figures 4C,D**). In the differentiating subgroup, the ARG signature failed to successfully stratify patients into two risk groups (**Figure 4E**),

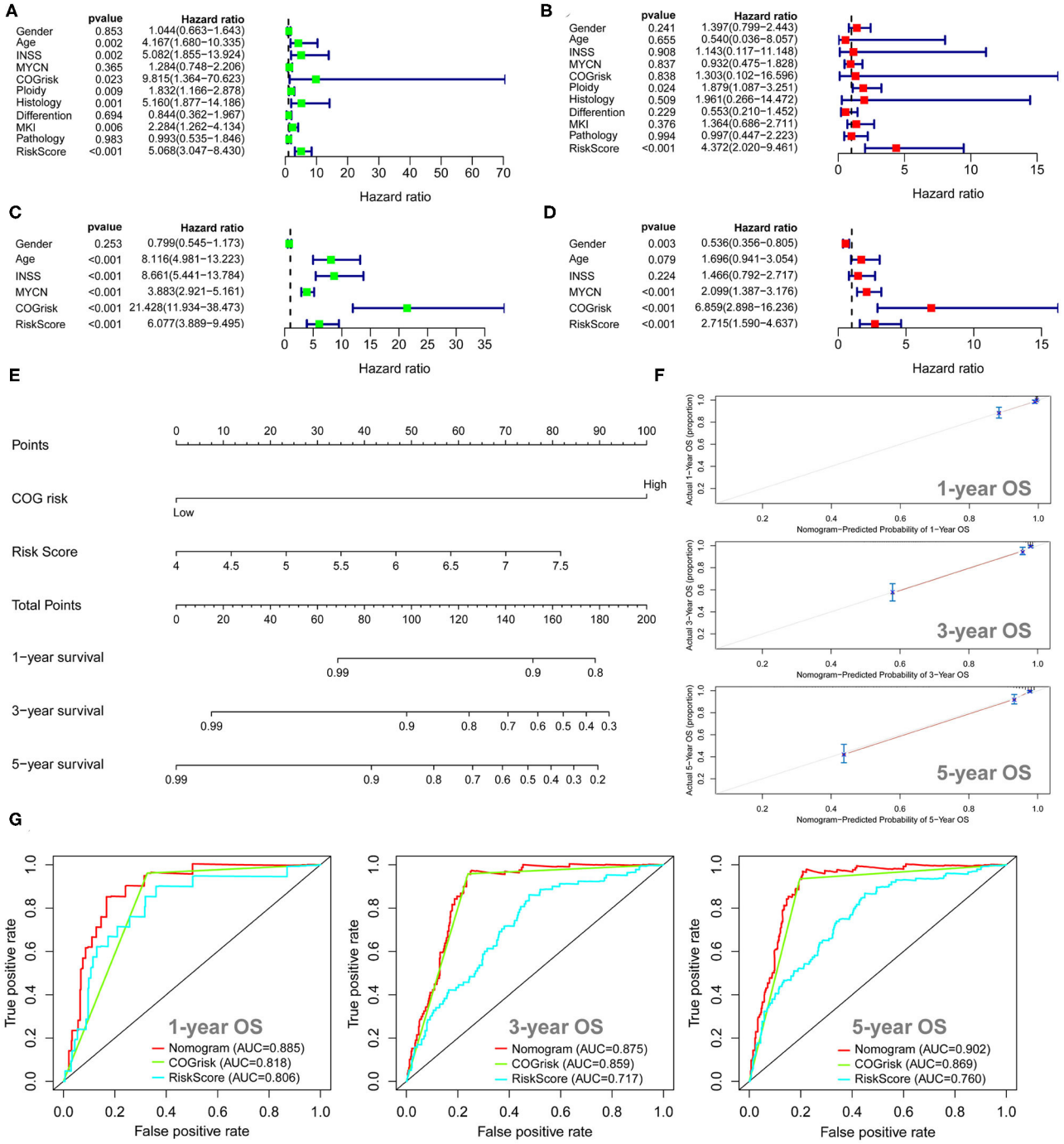


FIGURE 3 | Univariate and multivariate survival analyses of the autophagy-related gene (ARG) signature. **(A)** Univariate survival analysis of the ARG signature and other clinical risk factors in cohort 1. **(B)** Multivariate survival analysis of the ARG signature and other clinical risk factors in cohort 1. **(C)** Univariate survival analysis of the ARG signature and other clinical risk factors in cohort 2. **(D)** Multivariate survival analysis of the ARG signature and other clinical risk factors in cohort 2. **(E)** The nomogram model for prediction of overall survival in cohort 2. **(F)** The 1-, 3-, and 5-year calibration curves for the nomogram. **(G)** The 1-, 3-, and 5-year ROC curve analyses for the nomogram.

whereas in the poorly differentiated subgroup, patients in the high-risk group had significantly worse OS than patients in the low-risk group (**Figure 4F**). In both of the ploidy subtype (hyperdiploid and diploid), patients in the high-risk group had significantly worse OS than patients in the low-risk group (**Figures 4G,H**). In both the low MKI subgroup and intermediate

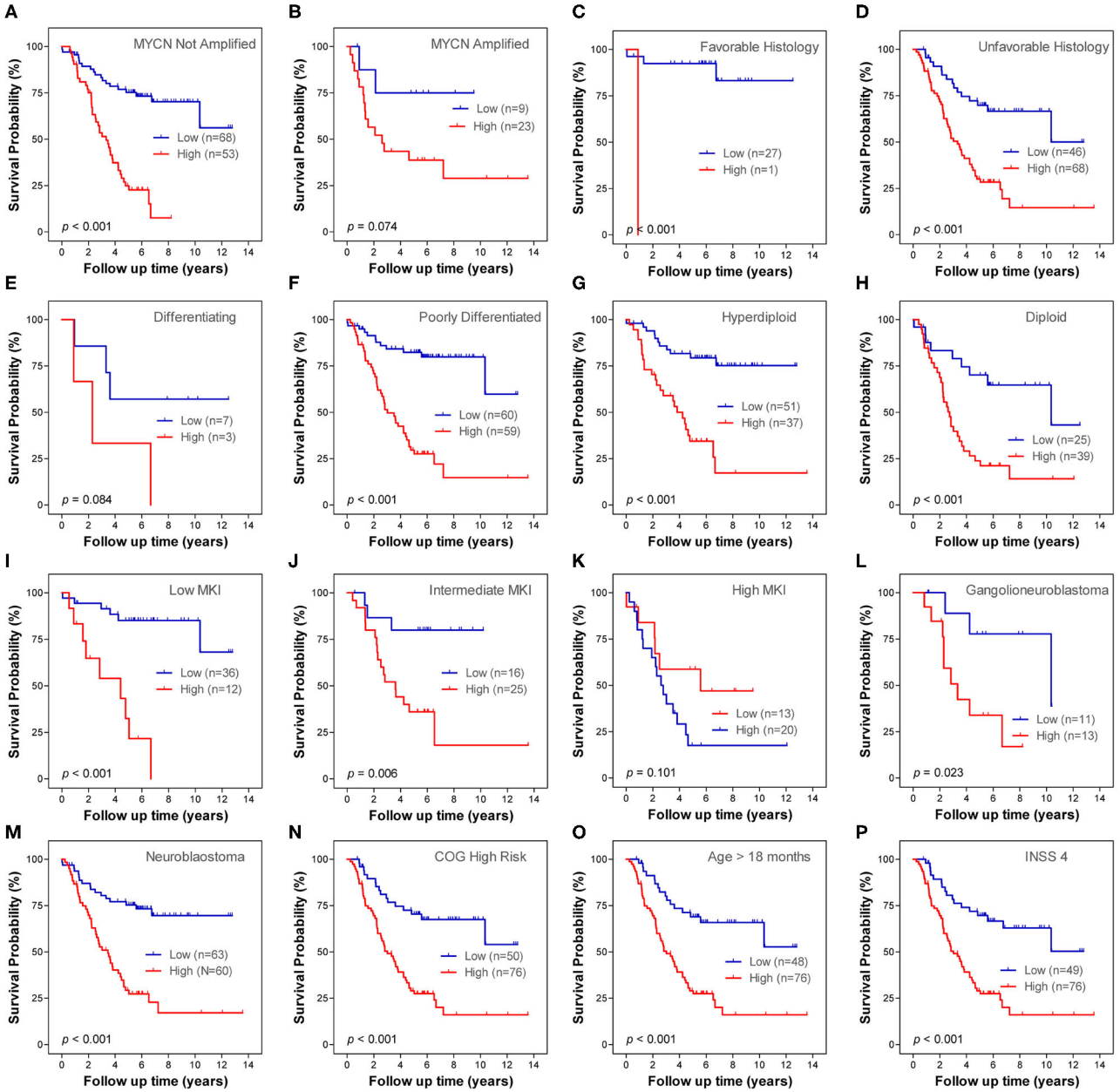


FIGURE 4 | Kaplan–Meier plots show the prognostic role of the autophagy-related gene (ARG) signature for overall survival in different subgroups of cohort 1. **(A)** MYCN not amplified. **(B)** MYCN amplified. **(C)** Favorable histology. **(D)** Unfavorable histology. **(E)** Differencing. **(F)** Poorly differentiated. **(G)** Hyperdiploid. **(H)** Diploid. **(I)** Low mitosis-karyorrhexis index (MKI). **(J)** Intermediate MKI. **(K)** High MKI. **(L)** Ganglioneuroblastoma. **(M)** Neuroblastoma. **(N)** Children's Oncology Group (COG) high risk. **(O)** Age > 18 months. **(P)** International Neuroblastoma Staging System (INSS) stage 4. The *p*-values were obtained using a Mantel log-rank test (two-sided).

MKI subgroup, patients with a high-risk score had a significantly worse OS than patients with a low-risk score (**Figures 4I,J**); however, the ARG signature failed to stratify patients in the high MKI subgroup into two risk groups (**Figure 4K**). In both of the ganglioneuroblastoma subgroup and NB subgroup, patients with a high-risk score had a significantly worse OS than those with a low-risk score (**Figures 4L,M**). All patients in the COG low-risk subgroup were classified as ARG low-risk group; thus, the Kaplan–Meier plot was not constructed, whereas the ARG risk score significantly stratify patients in the COG high-risk subgroup into two risk groups for OS (**Figure 4N**). All patients with diagnosis age < 18 months were classified as ARG low-risk group; thus, the Kaplan–Meier plot was not constructed, whereas the ARG risk score significantly stratify patients in the age > 18 month subgroup into two risk groups for OS (**Figure 4O**). All patients in stage 4s were classified as ARG low-risk group; thus,

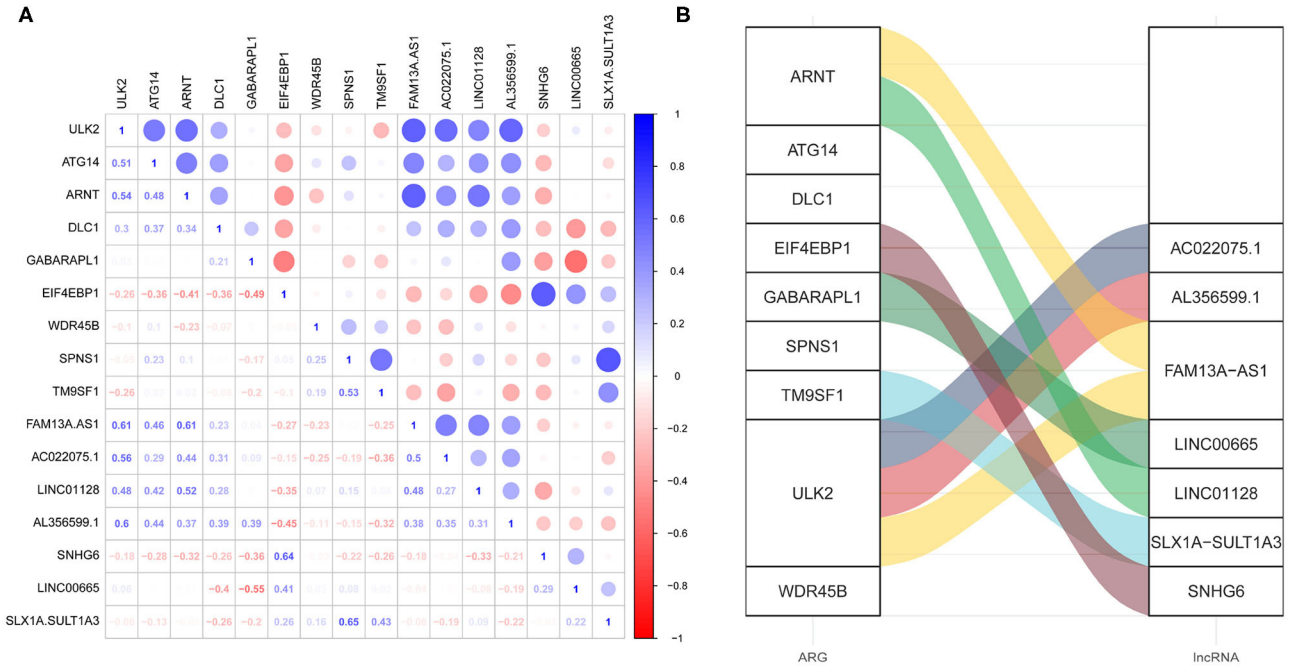


FIGURE 5 | The correlation between the autophagy-related genes (ARGs) and the autophagy-related long non-coding RNAs (lncRNAs). **(A)** The correlation matrix showing the Pearson correlation coefficients. **(B)** The alluvial diagram showing the correlation between the ARGs and the lncRNAs, which has a coefficient ≥ 5.

the Kaplan–Meier plot was not constructed, whereas the ARG risk score significantly stratifies patients in the stage 4 subgroup into two risk groups for OS (**Figure 4P**). There is only one patient classified as stage 2, six patients classified as stage 3, and no patients classified as stage 1 in cohort 1. Thus, we did not conduct subgroup analysis for stage 1, stage 2, and stage 3.

Construction and Validation of Autophagy-Related LncRNA Prognostic Signature

The lncRNAs correlated (Pearson correlation threshold ≥ 0.5) with the nine ARGs in the ARG signature were extracted as autophagy-related lncRNAs. A total of 562 autophagy-related lncRNAs were identified in cohort 1. However, only 18 autophagy-related lncRNA were shown to be significantly associated with OS by the univariate Cox survival analysis (**Supplementary Figure 3A**). The survival-related lncRNAs were put into LASSO Cox survival analysis to eliminate false positives. The 1 – SE criterion revealed no gene in the model; thus, the minimum lambda value was selected ($\lambda = 0.0419$) (**Supplementary Figures 3B,C**). Finally, seven autophagy-related lncRNAs (**Supplementary Table 3**) were selected and incorporated into the lncRNA signature risk score. The correlation between these seven lncRNAs and the ARGs is shown in **Figures 5A,B**. **Figure 5A** shows their Pearson correlation coefficients, whereas **Figure 5B** shows the links between the ARGs and lncRNAs, which have a coefficient ≥ 0.5.

The lncRNA signature risk scores were calculated for each patient as follows: risk score = 0.4820 * SLX1A – SULT1A3 + 0.0578 * LINC00665 + 0.0050 * SNH6 – 0.1992 * FAM13A-AS1 – 0.1984 * AC022075.1 – 0.0273 * LINC01228 – 0.0084 * AL356599.1. The median value was used as the cutoff value, and the entire cohort 1 was classified into two risk groups accordingly. The risk distribution, survival status, and gene expression pattern are shown in **Figure 6A**. The scatter plot (**Figure 6A**) shows that most of the patients in the high-risk group died and that most of the patients in the low-risk group survived during the 15-year follow-up. The heat map (**Figure 6A**) shows that four lncRNAs were highly expressed in the low-risk group whereas three lncRNAs were highly expressed in the high-risk group. Kaplan–Meier plots show that patients in the high-risk score group have a significantly worse OS than those in the low-risk score group (**Figure 6B**). The 3-, 5-, and 10-year OS rates for the patients in high-risk group were 50.39, 34.24, and 32.03%, respectively, whereas, the 3-, 5-, and 10-year OS rates for patients in low-risk group were 82.42, 72.16, and 66.96%, respectively. Time-dependent ROC curves reveal that the lncRNA signature has good performance in predicting OS in cohort 1, whereas the AUC at 3-, 5-, and 10-years was 0.77, 0.77, and 0.63, respectively (**Figure 6C**).

The lncRNA signature was tested in cohort 2 ($n = 498$) and cohort 3 ($n = 223$) for validation using the same risk score formula. According to the same cut-off value as cohort 1, the validation cohorts were divided into two risk groups. The risk distribution, survival status, and gene expression pattern for cohort 2 are shown in **Figure 6D**. Kaplan–Meier plots show

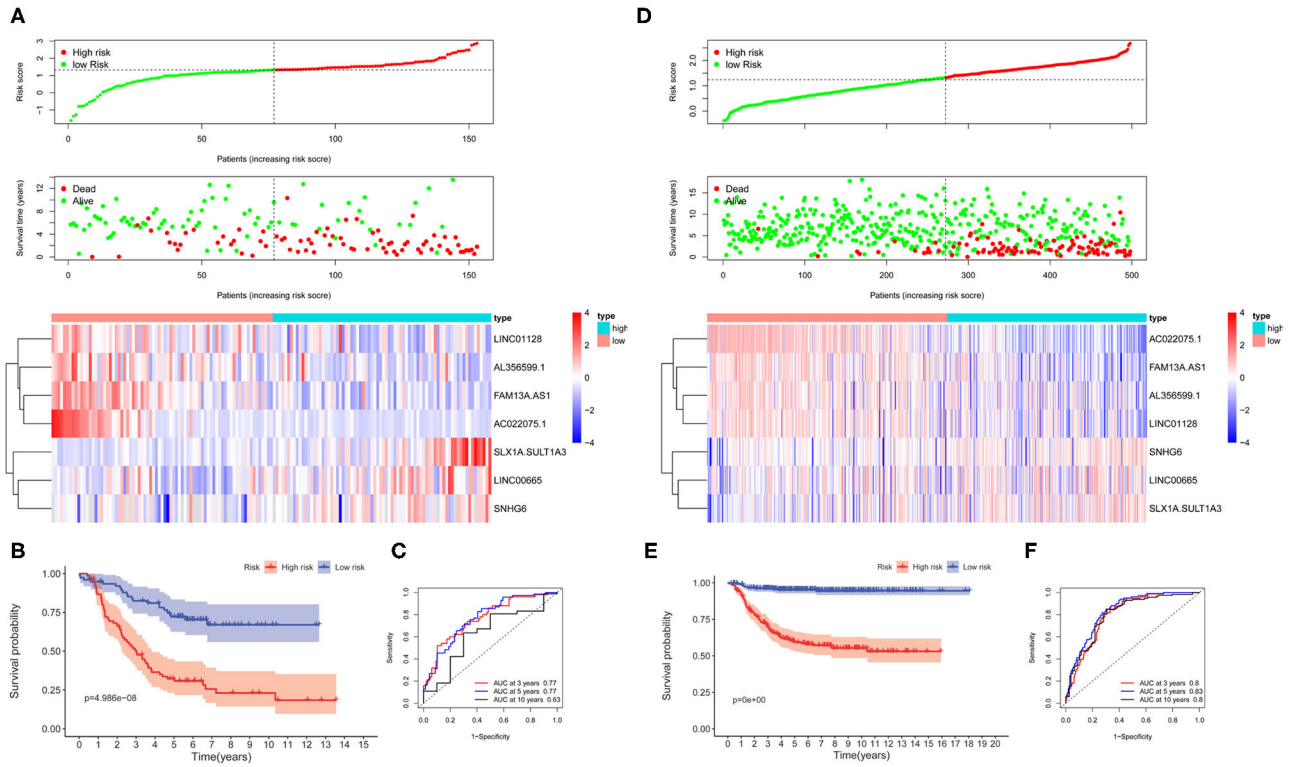


FIGURE 6 | The autophagy-related long non-coding RNA (lncRNA) signature risk score for neuroblastoma. **(A)** The distribution of risk scores, survival status of each patient, and heat map of lncRNA expression pattern in cohort 1. **(B)** Kaplan–Meier survival curve for overall survival (OS) of patients in the low-risk group and high-risk group for cohort 1. **(C)** Time-dependent receiver operating characteristic (ROC) curves for the prognostic value of the lncRNA signature in cohort 1. **(D)** The distribution of risk scores, survival status of each patient, and heat map of lncRNAs expression pattern in cohort 2. **(E)** Kaplan–Meier survival curve for OS of patients in the low-risk group and high-risk group for cohort 2. **(F)** Time-dependent ROC curves for the prognostic value of the lncRNA signature in cohort 2.

that patients in the high-risk score group have a significantly poorer OS than those in the low-risk score group in cohort 2 (**Figure 6E**). Time-dependent ROC curves reveal that the lncRNA signature has good performance in predicting OS in cohort 2, whereas the AUC at 3-, 5-, and 10-years was 0.8, 0.83, and 0.8, respectively (**Figure 6F**). Consistent with cohort 1 and cohort 2, the validation in cohort 3 shows similar results (**Supplementary Figure 4**).

Survival Analysis for the Autophagy-Related LncRNA Prognostic Signature

The univariate Cox regression survival analyses for the lncRNA signature risk sore and other clinical risk factors in cohort 1 are shown in **Figure 7A**. The lncRNA signature risk score is significantly associated with OS (HR = 3.976; 95%CI: 2.572–6.148; $p < 0.001$) in the univariate survival analysis. Multivariate Cox survival analyses including gender (female vs. male), age status (<18 vs. ≥18 months), INSS stage (INSS 2/3/4S vs. INSS 4), MYCN amplification (non-amplified vs. amplified), COG risk status (low risk vs. high risk), ploidy (hyperploid vs. diploid), histology type (favorable vs. unfavorable), differentiation (differentiating vs. poorly differentiated), MKI (low/intermediate vs. high), and pathology subtype (ganglioneuroblastoma vs. NB) as covariates were performed to evaluate the independent prognostic role of the lncRNA signature (**Figure 7B**). In cohort 1, only the lncRNA signature (HR = 6.186; 95%CI: 3.052–12.536, $p < 0.001$) and ploidy (HR = 2.139; 95%CI: 1.229–3.772; $p = 0.007$) were independently associated with OS (**Figure 7B**). The univariate and multivariate Cox regression survival analyses for the lncRNA signature risk sore and other clinical risk factors in cohort 2 are shown in **Figures 7C,D**. The lncRNA signature risk score is significantly associated with OS in cohort 2 by both univariate model (HR = 7.199; 95%CI: 4.763–10.881; $p < 0.001$) and multivariate model (HR = 2.005; 95%CI: 1.220–3.294; $p = 0.006$). We also built a nomogram incorporating the COG risk classification and the lncRNA signature risk score for prediction of OS on the basis of the largest cohort (cohort 2, $n = 498$) (**Figure 7E**). As is shown in the nomogram (**Figure 7E**), COG low risk was denoted as 0 point, whereas COG high risk was denoted as 79 points. As for the lncRNA signature risk score in the nomogram, a risk score of −0.5 was denoted as 0 point, and a risk score of 3 was denoted as 100 points. The risk scores between −0.5 and 3 were

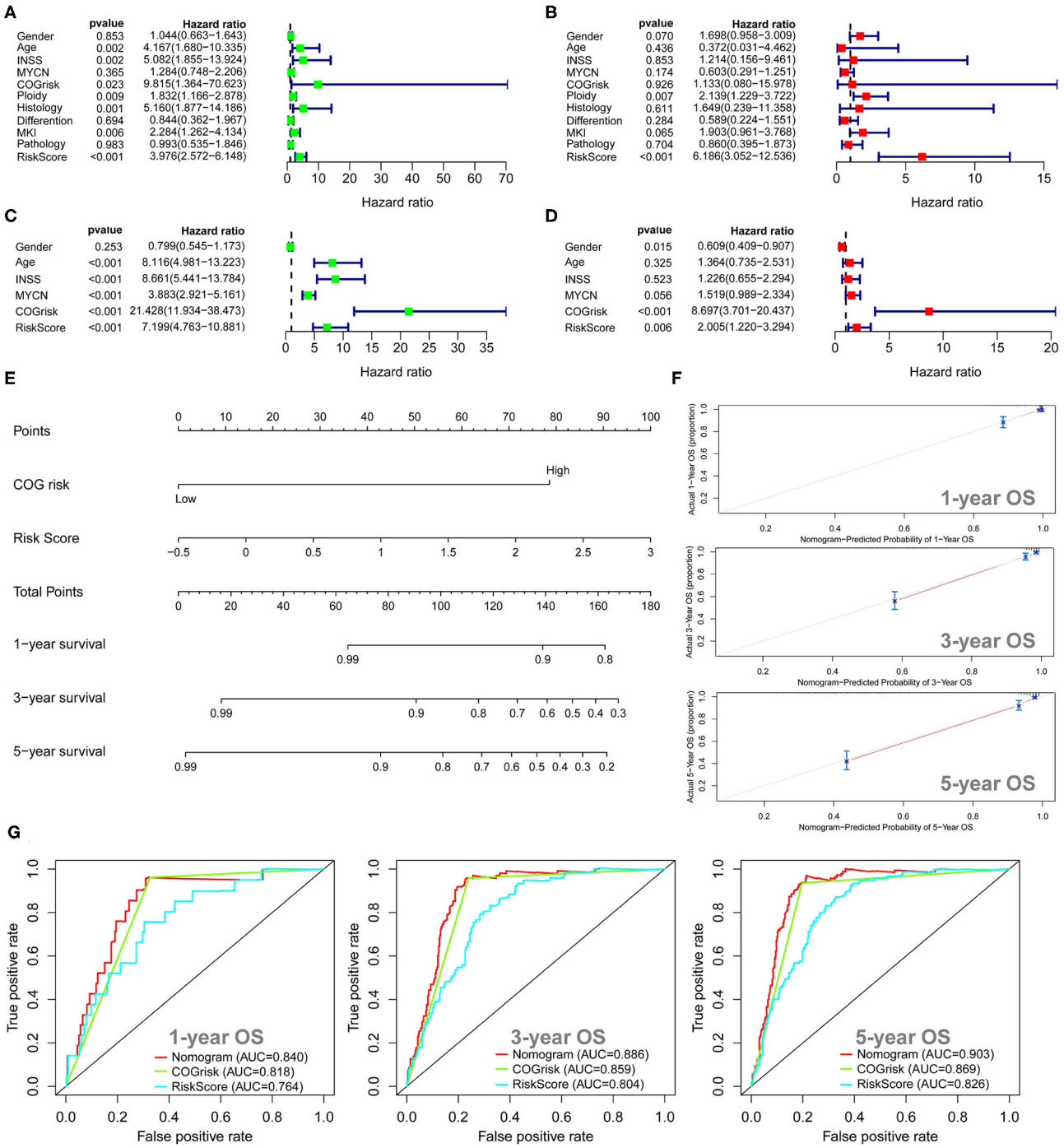


FIGURE 7 | Univariate and multivariate survival analyses for the autophagy-related long non-coding RNA (lncRNA) signature. **(A)** Univariate survival analysis of the lncRNA signature and other clinical risk factors in cohort 1. **(B)** Multivariate survival analysis of the lncRNA signature and other clinical risk factors in cohort 1. **(C)** Univariate survival analysis of the lncRNA signature and other clinical risk factors in cohort 2. **(D)** Multivariate survival analysis of the lncRNA signature and other clinical risk factors in cohort 2. **(E)** The nomogram model for prediction of overall survival in cohort 2. **(F)** The 1-, 3-, and 5-year calibration curves for the nomogram. **(G)** The 1-, 3-, and 5-year receiver operating characteristic (ROC) curve analyses for the nomogram.

assigned correspondingly between 0 and 100 points and could be calculated as follows: points = (risk score + 0.5) * (100/3.5). The total points for the patients were calculated by combining the points for COG risk and the points for the lncRNA risk score. The corresponding predicted survival probability is shown below.

The *C*-index for the nomogram was 0.85 (95%CI: 0.81–0.88), indicating a high level of accuracy. The 1-, 3-, and 5-year calibrate

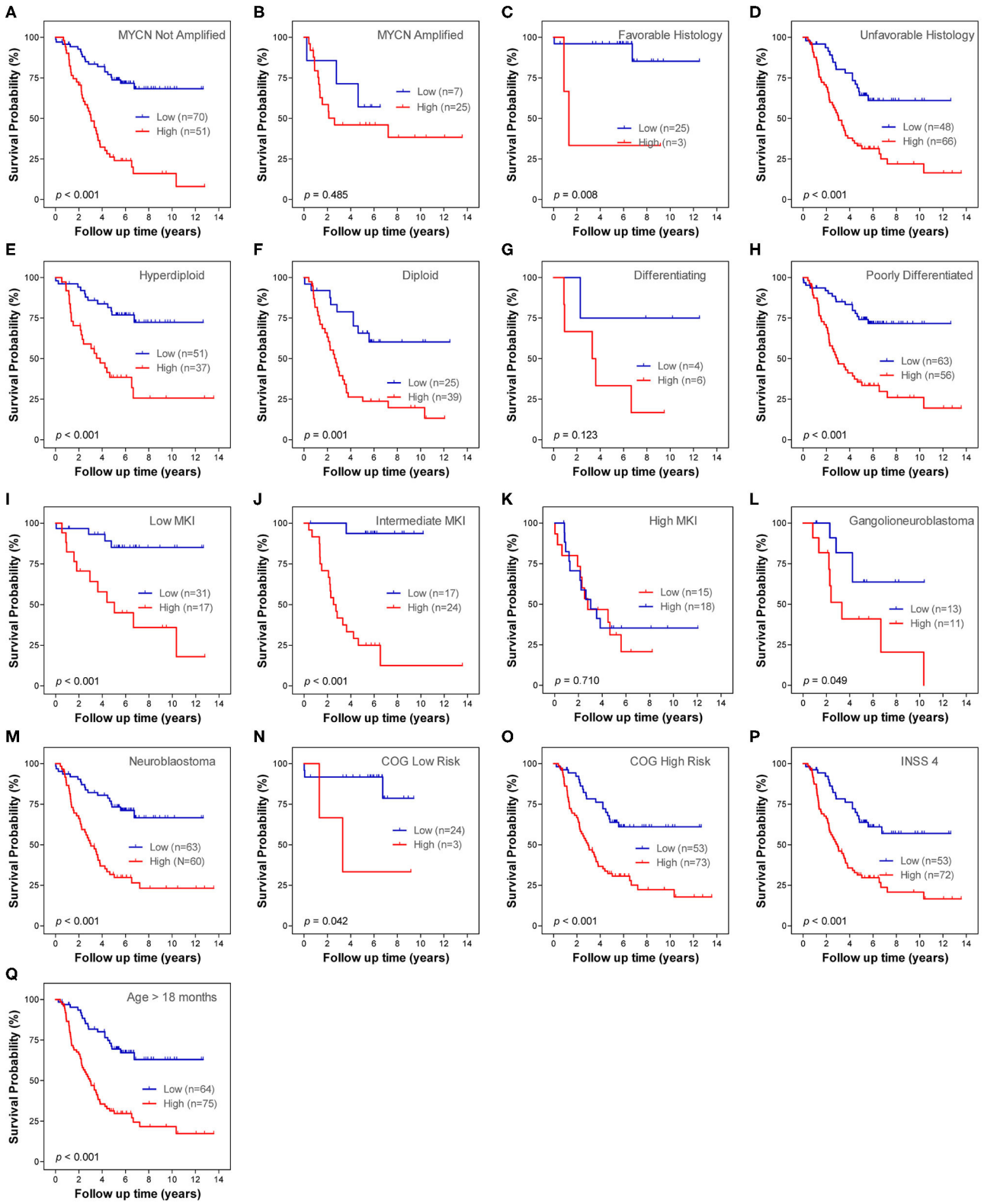
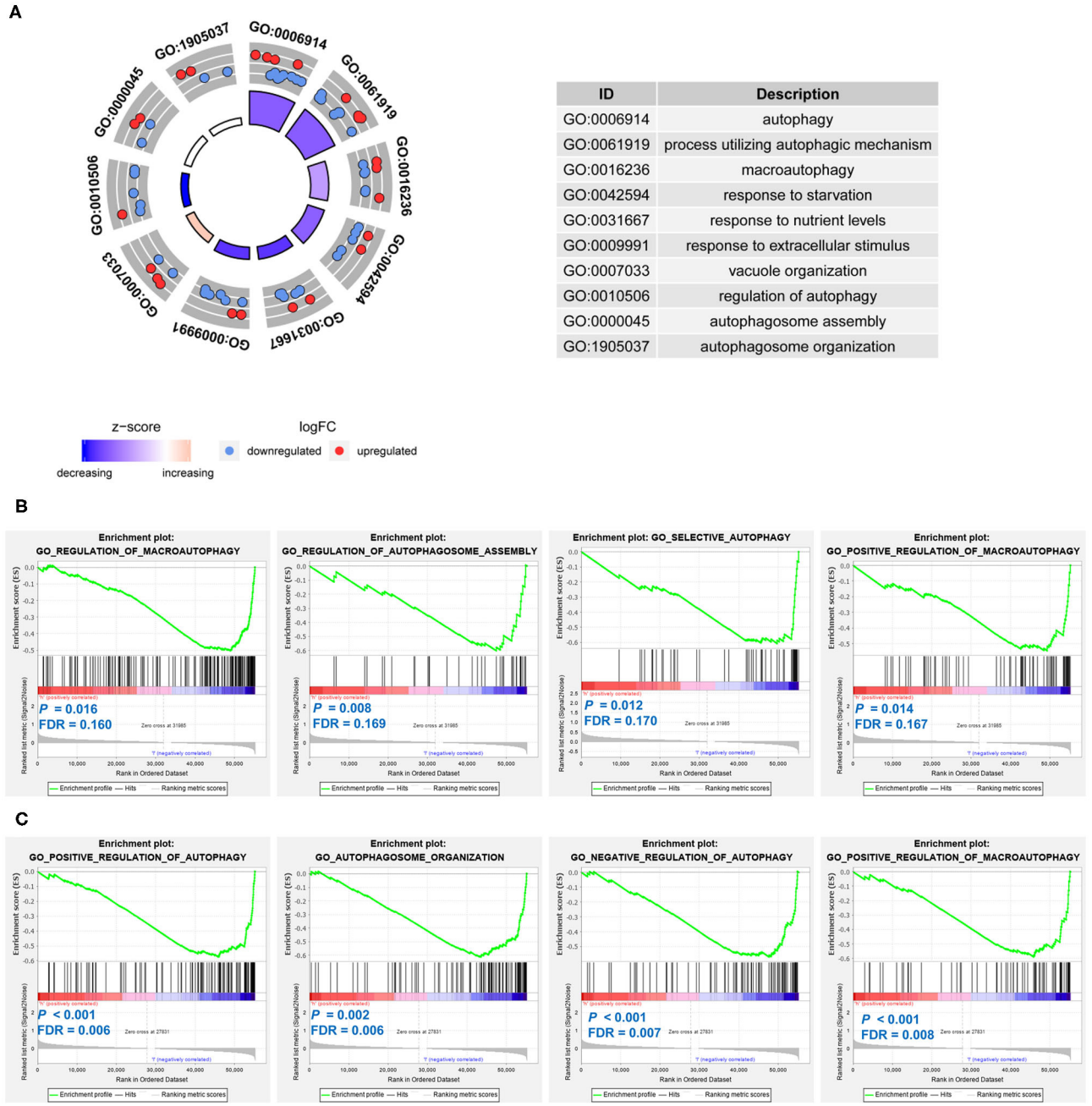


FIGURE 8 | Kaplan–Meier plots show the prognostic role of the long non-coding RNA (lncRNA) signature for overall survival in different subgroups of cohort 1. **(A)** MYCN not amplified. **(B)** MYCN amplified. **(C)** Favorable histology. **(D)** Unfavorable histology. **(E)** Differencing. **(F)** Poorly differentiated. **(G)** Hyperdiploid. **(H)** Diploid. **(I)** Low mitosis-karyorrhexis index (MKI). **(J)** Intermediate MKI. **(K)** High MKI. **(L)** Ganglioneuroblastoma. **(M)** Neuroblastoma. **(N)** Children's Oncology Group (COG) low risk. **(O)** COG high risk. **(P)** International Neuroblastoma Staging System (INSS) stage 4. **(Q)** Age > 18 months. The *p*-values were obtained using a Mantel log-rank test (two-sided).



ID	Description
GO:0006914	autophagy
GO:0061919	process utilizing autophagic mechanism
GO:0016236	macroautophagy
GO:0042594	response to starvation
GO:0031667	response to nutrient levels
GO:0009991	response to extracellular stimulus
GO:0007033	vacuole organization
GO:0010506	regulation of autophagy
GO:0000045	autophagosome assembly
GO:1905037	autophagosome organization

FIGURE 9 | Function annotation and Gene Set Enrichment Analyses (GSEAs) of the prognostic signatures in neuroblastoma. **(A)** The circle plot of Gene Ontology (GO) function annotation for the differentially expressed and survival-related autophagy-related genes (ARGs). **(B)** Gene sets enriched in the low-risk group of the ARG signature. **(C)** Gene sets enriched in the low-risk group of the autophagy-related long non-coding RNA (lncRNA) signature.

curves for the nomogram all revealed that the predicted OS was very close to the actual OS (**Figure 7F**). The ROC curves analyses revealed that the AUC values at 1-, 3-, and 5-years for the nomogram were higher than the AUC values at 1-, 3-, and 5-years for the COG risk, respectively (**Figure 7G**), indicating that the prognostic role of the nomogram is more accurate than the COG risk classification alone.

Prognostic Role of the Autophagy-Related LncRNA Signature Within Clinical Subgroups

Stratification survival analyses were performed in order to evaluate the prediction ability of the lncRNA signature in different clinical subgroups. The subgroups were classified according to MYCN amplification status (not

amplified and amplified), histology subtype (favorable and unfavorable), differentiation status (differentiating and poorly differentiated), ploidy status (hyperdiploid and diploid), MKI status (low, intermediate and high), pathology subtype (ganglioneuroblastoma and NB), COG risk status (low and high), age status (age $<$ 18 months and age $>$ 18 months), and INSS stage. Within each subgroup, patients were classified into low-risk and high-risk subgroups on the basis of the same cutoff value from the entire cohort 1. Except for MYCN amplified subgroup, differentiating subgroup and high MKI subgroup, the lncRNA signature risk score significantly stratifies patients into two risk groups for OS in all of the other subgroups (**Figure 8**). Only one patient in stage 4s was classified as lncRNA high-risk group; thus, the Kaplan–Meier plot was not constructed. There is only one patient classified as stage 2, six patients classified as stage 3, and no patients classified as stage 1 in cohort 1. Thus, we did not conduct subgroup analysis for stage 1, stage 2, and stage 3.

Gene Ontology Function Annotation and Gene Set Enrichment Analyses for the Prognostic Signatures

The 21 differentially expressed and survival-related ARGs were put into GO functional annotation. The circle plot of GO revealed that autophagy biological processes (GO: 0006914 autophagy, GO: 0061919 process utilizing autophagic mechanism, GO: 0016236 macroautophagy, and GO: 0010506 regulation of autophagy) were down-regulated in stage 4 NB (**Figure 9A**).

GSEAs were also conducted to compare the difference between low-risk groups and high-risk groups. For both of the ARG signature and lncRNA signature, no autophagy-related gene set was enriched in the high-risk groups. Gene sets of GO regulation of macroautophagy, GO regulation of autophagosome assembly, GO selective autophagy, and GO positive regulation of macroautophagy were significantly enriched in the low-risk group of the ARG signature (**Figure 9B**). Gene sets of GO positive regulation of autophagy, GO autophagosome organization, GO negative regulation of autophagy, and GO positive regulation of macroautophagy were significantly enriched in the low-risk group of the lncRNA signature (**Figure 9C**).

Genetic Alterations of the Genes in the Prognostic Signatures

The cBioportal platform was used to explore the genetic alterations of the nine ARGs and the seven lncRNAs in NB tumors (**Supplementary Figure 5**). The mutation data in 755 NB tissue samples and the somatic gene copy number data in 59 NB tissue samples were provided by cBioportal. The results showed that MYCN gene has somatic gene copy number alteration in about 19% of the NB tissue samples and has mutations in about 1.2% of the NB tissue samples. Only GABARAPL1 was found to have amplification in 1.7% of NB samples (**Supplementary Figure 5A**). SPNS1, DLC1, and ARNT were found to have missense mutation in 0.1% of NB samples (**Supplementary Figure 5B**). No gene alteration data were available for the lncRNA AC0022075.1 and AL356599.1. No somatic gene copy number alteration or mutation was detected for each of the other lncRNAs (**Supplementary Figures 5C,D**).

DISCUSSION

Autophagy is a highly conserved homeostatic pathway, which captures intracellular proteins and organelles and put them into degradation and recycling (21, 22). The role of autophagy in cancer is context dependent; in some models, autophagy could suppress cancer genesis, whereas some cancers are dependent on autophagy for survival (22). Some researchers reported the tumor-suppressive role of autophagy for NB. For example, one study reported that inhibition of cyclooxygenase-2 (COX-2) promotes 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced autophagic cell death in human NB cell line SH-SY5Y (29); another study revealed that calcium/calmodulin-dependent protein kinase II (CAMK2) promotes autophagic degradation of inhibitor of differentiation 1/2 (Id-1/2) and then induces cell differentiation in NB (24). However, there are also studies that found the tumor-protective role of autophagy for NB. For example, rapid induction of ARG GABARAPL1 could promote NB cell survival before autophagy activation (25); autophagy was also found to be associated with chemoresistance of NB (26).

The association between ARGs and the spontaneous regression of NB is largely unknown. To our knowledge, this present study is the first with the purpose finding out ARGs associated with spontaneous regression of NB by combining both RNA-Seq and microarray data. Because spontaneous regression of NB is most prevalent in stage 4S NB patients, in this study, stage 4s tumors were used as surrogates to explore the underlining mechanisms responsible for the spontaneous regression of NB as many other investigators have done before. The dead cases in stage 4s were excluded to make it better to serve as surrogates. Actually, there were only two out of 21 stage 4s cases who died in cohort 1, five out of 54 stage 4s cases who died in cohort 2, and one out of 30 stage 4s cases who died in cohort 3 during more than 10-years of follow-up.

In this study, out of 233 ARGs in the Human Autophagy Database, a total of 48 ARGs were found to be differentially expressed between stage 4s and stage 4 NB samples, and 19 of these 48 ARGs were found to be significantly correlated with OS of NB patients. After LASSO Cox survival analysis, nine ARGs were found to have the best prognostic value and were used to construct an ARG prognostic signature. The ARG signature risk score successfully divided each of the cohorts into two different risk groups, with the low-risk group having good survival outcome and the high-risk group having bad survival outcome. The ARG signature also performed well in the subgroup survival analyses on the basis of different clinical risk factor stratifications. Multivariate survival analyses revealed that the prognostic role of this ARG signature is independent with other clinical risk factors. These results corroborate the role of

autophagy in the genesis and progression of NB and suggest the use of this ARG signature as a risk factor for risk stratification.

Most NB patients in North America are treated according to the COG risk classification system. Based on MYCN amplification status, age at diagnosis, INSS stage, histopathology, and tumor cell ploidy, NB patients are stratified into low-, intermediate-, and high-risk groups according to the 2007 COG risk system (30, 31). The latest available data reveal that the 5-year OS rate was about 97% for COG low-risk NB patients (32); the 3-year OS rate was about 96% for COG intermediate-risk NB patients (33); and the OS rate for COG high-risk NB patients is only about 50% (31). In this study, although all patients in the COG low-risk subgroup were also classified as ARG signature low risk, the ARG signature significantly striated patients in the COG high-risk NBs into two risk groups. This suggests that the ARG signature risk score could improve the prognostic ability of COG risk classification system. We thus built a nomogram on the basis of the COG risk classification and the ARG signature risk score using the largest cohort (cohort 2, $n = 498$), which shows good accuracy for OS prediction. One drawback is that this dataset (cohort 2, GSE49710) only consists of COG low-risk and high-risk NB patients, whereas no NB patients were in the intermediated-risk group. In the TAGERT NBL cohort ($n = 153$), there are only several cases classified as COG intermediate risk, and we thus combined COG intermediate-risk and low-risk NBs together as one group during the analysis. However, because the OS rate of COG low-risk NB patients and COG intermediate-risk NB patients is similar (32, 33), we think that the influence of this drawback is limited.

The nine ARGs incorporated in the ARG signature include SPNS1 (sphingolipid transporter 1), TM9SF1 (transmembrane 9 superfamily member 1), WDR45B (WD repeat domain 45B), EIF4EBP1 (eukaryotic translation initiation factor 4E binding protein 1), GABARAPL1 (gamma-aminobutyric acid receptor-associated protein-like 1), ATG14 (autophagy related 14), ULK2 (unc-51 like kinase 2), DLC1 (DLC1 Rho GTPase activating protein), and ARNT (aryl hydrocarbon receptor nuclear translocator). Five (ATG4, ULK2, ARNT, GABARAPL1, and DLC1) of them are highly expressed in the low-risk group and are associated with good OS, whereas four (EIF4EBP1, WDR45B, SPNS1, and TM9SF1) of them are highly expressed in the high-risk group and are associated with bad OS.

The five ARGs that associated good survival of NB are all important positive regulators of autophagy. Two of them (ULK1 and ARNT) have been found to have important roles in regulating neuronal development: ULK1 is essential to mediate autophagy under nutrient-deficient conditions and regulate axon guidance in the developing forebrain of mouse via a non-canonical pathway (34, 35); ARNT is mostly expressed in neuronal cell types and play roles in regulating dendritic morphology and neuronal differentiation (36). Two of them (GABARAPL1 and DLC1) have been found to have a tumor-suppressive function: the GABARAPL1 protein could positively regulate ULK1 activity and autophagosome formation (37) and was also found to have a tumor-suppressive function in breast cancer cells (38, 39); DLC1 is involved in regulating autophagy and apoptosis and was found to be a potential tumor suppressor in many types of human cancers (40). ATG4 is the only protease functions as an important factor in the ATG8 conjugation system, and its activity is essential to autophagy (41). It has to be mentioned that one study revealed that rapid induction of GABARAPL1 promotes NB cell survival before autophagy activation (25), which is somewhat inconsistent with our findings as GABARAPL1 was found to be associated with good survival in our study. However, as is described in the literature, the protective role of GABARAPL1 for NB cell functions before autophagy activation (25).

The function of the four ARGs that associated bad survival of NB in our study has been reported as follows: EIF4EBP1 is a downstream target of mTOR signaling pathway and could inhibit autophagy initiation (42, 43); WDR45B was found to play an essential role in maintaining neural autophagy and neural homeostasis (44); SPNS1 was found to play an important role in orchestrating autolysosomal biogenesis and is critically linked to developmental senescence and survival (45); TM9SF1 was found to play important roles in inducing autophagy (46). Except for GABARAPL1, the roles of the other eight ARGs in NB genesis and progression have not been reported. The exact roles of these ARGs in NB and their underlining mechanisms need to be investigated by further studies.

LncRNAs are known as RNA transcripts longer than 200 nucleotides with no protein-coding capacity (47). LncRNAs are crucial players in various types of cancers including NB (48–52). In this study, we identified that the expression of 18 survival-related lncRNAs are correlated with the expression of the nine ARGs in the ARG signature. We termed these lncRNAs as autophagy-related lncRNAs. Seven autophagy-related lncRNAs were identified as having the best prognostic value by the LASSO Cox survival analyses. These seven autophagy-related lncRNAs were used to construct an autophagy-related lncRNA signature. The lncRNA signature risk score also successfully divided each of the cohorts into two different risk groups, with the low-risk groups having good survival outcome and the high-risk groups having bad survival outcome. The lncRNA signature performed well in the subgroup survival analyses on the basis of different clinical risk factor stratifications. Multivariate survival analyses revealed that the prognostic role of this lncRNA signature is also independent with other clinical risk factors. Different from the ARG signature, this lncRNA signature significantly stratified both COG low-risk NBs and COG high-risk NBs into two risk groups, indicating a somewhat better prediction accuracy. The *C*-index for the nomogram based on the lncRNA signature is a little higher than the *C*-index for the nomogram based on the ARG signature (0.85 vs. 0.84).

These seven lncRNAs incorporated in the lncRNA signature include FAM13A-AS1, SLX1A-SULT1A3, SNHG6, LINC001128, LINC00665, AL356599.1, and AC022075.1. Four (LINC01128, FAM13A-AS1, AL356599.1, and AC022075.1) of them are highly expressed in the low-risk group and are associated with good OS, whereas three (SLX1A-SULT1A3, LINC00665, and SNHG6) of them are highly expressed in the high-risk group and are associated with bad OS. The function of these lncRNAs is largely unknown. SNHG6 has been found to function by sponging microRNAs and to act as an oncogene in gastric cancer, colorectal

cancer, and lung adenocarcinoma (53–55). LINC00128 could promote cervical cancer progression by binding with miR-383-5p and up-regulating Stratifin (56). The function of the other lncRNAs and their relation with cancer have not been reported in the literature. The relation of these lncRNA with autophagy, the role of their function in NB, and the underlying mechanisms still need to be clarified by further researches.

Investigation of the cBioportal platform discovered that GABARAPL1 has amplification in 1.7% of NB samples, whereas SPNS1, DLC1, and ARNT have missense mutation in 0.1% of NB samples. There is no somatic gene copy number alteration or mutation detected for these lncRNAs. It seems that genetic alterations play little roles in their differential expression in NB. However, this result should be interpreted with caution, as the number of NB cases providing genetic alteration information is limited. In addition, genetic alterations outside those genes might also be potential causes responsible for their altered expression. More studies are needed to figure out whether these ARGs and autophagy-related lncRNAs have genetic alterations in NB.

GO function annotation revealed that autophagy biological processes (GO: 0006914 autophagy, GO: 0061919 process utilizing autophagic mechanism, GO: 0016236 macroautophagy, and GO: 0010506 regulation of autophagy) were down-regulated in stage 4 NB. Consistent with the finding of GO function annotation, the GSEAs also revealed that autophagy gene sets were significantly enriched in the low-risk group: gene sets of GO regulation of macrophagy, GO regulation of autophagosome assembly, GO selective autophagy, and GO positive regulation of macroautophagy were significantly enriched in the low-risk group of the ARG signature, whereas gene sets of GO positive regulation of autophagy, GO autophagosome organization, GO negative regulation of autophagy, GO positive regulation of macroautophagy were significantly enriched in the low-risk group of the lncRNA signature. It is very interesting to find that no autophagy gene set is enriched in the high-risk groups. These results suggest that autophagy might mainly play a tumor-suppressive role in NB and might be associated with the spontaneous regression of NB. Undoubtedly, further investigations are needed to clarify how autophagy affects the process of spontaneous regression.

There are indeed some drawbacks in this study. Firstly, we did not perform *in vivo* or *in vitro* experimental studies to corroborate the findings of the present study. The exact roles of these identified ARGs or lncRNA in NB are largely unknown. Their underlining mechanisms responsible for NB progression or regression need to be clarified by further experimental studies. Secondly, spontaneous regression of NB did not occur in stage 4s tumors only, and not all cases in stage 4s underwent spontaneous regression. However, many other researchers have used stage 4s tumors as a surrogate. The dead cases in stage 4s NBs were also excluded in this study to make it better to serve as surrogates. Thirdly, the prognostic role of the signatures in some subgroups stratified by clinical risk factors showed no statistical significance. We think that the main reason is the low case number in these subgroups. Studies with larger sample size for these subgroups are needed. Despite these drawbacks, the combination of RNA-Seq data and microarray data, the large sample size of the three cohorts, and the validation of the findings by two independent cohorts all provide a high level of confidence.

In conclusion, we find that ARGs are differentially expressed between the stage 4 and stage 4s NB samples. The ARG prognostic signature has good performance in predicting OS of NB patients. The autophagy-related lncRNA signature also has good performance in predicting OS of NB patients. The prognostic value of both the ARG signature and lncRNA signature is independent of other clinical risk factors. The autophagy-related signatures have the potential to be used as risk factors for risk stratification of NB. Autophagy biological processes are significantly enriched in the low-risk groups and might mainly play a tumor-suppressive role in NB.

DATA AVAILABILITY STATEMENT

The dataset TARGET NBL was downloaded from https://portal.gdc.cancer.gov; the dataset GEO49710 was downloaded from https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE49710; the dataset E-MTAB-8248 was downloaded from https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-8248/.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

This study was designed by XZ. Data were analyzed by XM, HL, EF, and XZ. The original manuscript was written by XM and HL. Supervision and manuscript revision were performed by XZ and JF. Funding was acquired by XZ and JF. All authors read and approved the final manuscript.

FUNDING

This research was supported by the National Natural Science Foundation of China (grant number 81702472) and the Sanming Project of Medicine in Shenzhen (grant number SZSM201812055).

ACKNOWLEDGMENTS

We wish to thank Xinmei Nie for her English language support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.01411/full#supplementary-material

REFERENCES

1. Challis GB, Stam HJ. The spontaneous regression of cancer. A review of cases from 1900 to 1987. *Acta Oncol.* (1990) 29:545–50. doi: 10.3109/02841869009090048
2. Oquinena S, Guillen-Grima F, Inarrairaegui M, Zozaya JM, Sangro B. Spontaneous regression of hepatocellular carcinoma: a systematic review. *Eur J Gastroenterol Hepatol.* (2009) 21:254–7. doi: 10.1097/MEG.0b013e328324b6a2
3. Brodeur GM. Spontaneous regression of neuroblastoma. *Cell Tissue Res.* (2018) 372:277–86. doi: 10.1007/s00441-017-2761-2
4. Cervinkova M, Kucerova P, Cizkova J. Spontaneous regression of malignant melanoma - is it based on the interplay between host immune system and melanoma antigens? *Anticancer Drugs.* (2017) 28:819–30. doi: 10.1097/CAD.0000000000000526
5. Kumar T, Patel N, Talwar A. Spontaneous regression of thoracic malignancies. *Respir Med.* (2010) 104:1543–50. doi: 10.1016/j.rmed.2010.04.026
6. Mizuno T, Usami N, Okasaka T, Kawaguchi K, Okagawa T, Yokoi K. Complete spontaneous regression of non-small cell lung cancer followed by adrenal relapse. *Chest.* (2011) 140:527–8. doi: 10.1378/chest.10-2564
7. Meng X, Fang E, Zhao X, Feng J. Identification of prognostic long noncoding RNAs associated with spontaneous regression of neuroblastoma. *Cancer Med.* (2020) 9:3800–15. doi: 10.1002/cam4.3022
8. Suita S, Zaizen Y, Sera Y, Takamatsu H, Mizote H, Ohgami H, et al. Mass screening for neuroblastoma: Quo Vadis? A 9-year experience from the Pediatric Oncology Study Group of the Kyushu area in Japan. *J Pediatr Surg.* (1996) 31:555–8. doi: 10.1016/S0022-3468(96)90495-9
9. Bessho F. Comparison of the incidences of neuroblastoma for screened and unscreened cohorts. *Acta Paediatr.* (1999) 88:404–6. doi: 10.1111/j.1651-2227.1999.tb01130.x
10. Erttmann R, Tafese T, Berthold F, Kerbl R, Mann J, Parker L, et al. 10 years' neuroblastoma screening in Europe: preliminary results of a clinical and biological review from the Study group for evaluation of neuroblastoma screening in Europe (SENSE). *Eur J Cancer.* (1998) 34:1391–7. doi: 10.1016/S0959-8049(98)00135-X
11. Woods WG, Gao RN, Shuster JJ, Robison LL, Bernstein M, Weitzman S, et al. Screening of infants and mortality due to neuroblastoma. *N Engl J Med.* (2002) 346:1041–6. doi: 10.1056/NEJMoa012387
12. Lavarino C, Cheung NK, Garcia I, Domenech G, de Torres C, Alaminos M, et al. Specific gene expression profiles and chromosomal abnormalities are associated with infant disseminated neuroblastoma. *BMC Cancer.* (2009) 9:44. doi: 10.1186/1471-2407-9-44
13. Yu F, Zhu X, Feng C, Wang T, Hong Q, Liu Z, et al. Proteomics-based identification of spontaneous regression-associated proteins in neuroblastoma. *J Pediatr Surg.* (2011) 46:1948–55. doi: 10.1016/j.jpedsurg.2011.06.024
14. Benard J, Raguenez G, Kauffmann A, Valent A, Ripoche H, Joulin V, et al. MYCN-non-amplified metastatic neuroblastoma with good prognosis and spontaneous regression: a molecular portrait of stage 4S. *Mol Oncol.* (2008) 2:261–71. doi: 10.1016/j.molonc.2008.07.002
15. Evans AE, D'Angio GJ, Randolph J. A proposed staging for children with neuroblastoma. Children's Cancer Study group A. *Cancer.* (1971) 27:374–8.
16. Brodeur GM, Pritchard J, Berthold F, Carlsen NL, Castel V, Castelberry RP, et al. Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. *J Clin Oncol.* (1993) 11:1466–77. doi: 10.1200/JCO.1993.11.8.1466
17. Cohn SL, Pearson AD, London WB, Monclair T, Ambros PF, Brodeur GM, et al. The International Neuroblastoma Risk Group (INRG) classification system: an INRG task force report. *J Clin Oncol.* (2009) 27:289–97. doi: 10.1200/JCO.2008.16.6785
18. Nickerson HJ, Matthay KK, Seeger RC, Brodeur GM, Shimada H, Perez C, et al. Favorable biology and outcome of stage IV-S neuroblastoma with supportive care or minimal therapy: a Children's Cancer Group study. *J Clin Oncol.* (2000) 18:477–86. doi: 10.1200/JCO.2000.18.3.477
19. Tas ML, Nagtegaal M, Kraal KCJM, Tytgat GAM, Abeling NGGM, Koster J, et al. Neuroblastoma stage 4S: tumor regression rate and risk factors of progressive disease. *Pediatr Blood Cancer.* (2020) 67:e28061. doi: 10.1002/pbc.28061
20. Hero B, Simon T, Spitz R, Ernestus K, Gnekow AK, Scheel-Walter H-G, et al. Localized infant neuroblastomas often show spontaneous regression: results of the prospective trials NB95-S and NB97. *J Clin Oncol.* (2008) 26:1504–10. doi: 10.1200/JCO.2007.12.3349
21. Chen P, Cescon M, Bonaldo P. Autophagy-mediated regulation of macrophages and its applications for cancer. *Autophagy.* (2014) 10:192–200. doi: 10.4161/auto.26927
22. White E, Mehnert JM, Chan CS. Autophagy, metabolism, and cancer. *Clin Cancer Res.* (2015) 21:5037–46. doi: 10.1158/1078-0432.CCR-15-0490
23. Dower CM, Bhat N, Gebru MT, Chen L, Wills CA, Miller BA, et al. Targeted inhibition of ULK1 promotes apoptosis and suppresses tumor growth and metastasis in neuroblastoma. *Mol Cancer Ther.* (2018) 17:2365–76. doi: 10.1158/1535-7163.MCT-18-0176
24. Li X, Wu XQ, Deng R, Li DD, Tang J, Chen WD, et al. CaMKII-mediated Beclin 1 phosphorylation regulates autophagy that promotes degradation of Id and neuroblastoma cell differentiation. *Nat Commun.* (2017) 8:1159. doi: 10.1038/s41467-017-01272-2
25. Wang T, Liu L, Chen X, Shen Y, Lian G, Shah N, et al. MYCN drives glutaminolysis in neuroblastoma and confers sensitivity to an ROS augmenting agent. *Cell Death Dis.* (2018) 9:220. doi: 10.1038/s41419-018-0295-5
26. Belounis A, Nyalendo C, Le Gall R, Imbriglio TV, Mahma M, Teira P, et al. Autophagy is associated with chemoresistance in neuroblastoma. *BMC Cancer.* (2016) 16:891. doi: 10.1186/s12885-016-2906-9
27. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov.* (2012) 2:401–4. doi: 10.1158/2159-8290.CD-12-0095
28. Tibshirani R. The Lasso method for variable selection in the Cox model. *Stat Med.* (1997) 16:385–95.
29. Niranjan R, Mishra KP, Thakur AK. Inhibition of cyclooxygenase-2 (COX-2) initiates autophagy and potentiates MPTP-induced autophagic cell death of human neuroblastoma cells, SH-SY5Y: an inside in the pathology of Parkinson's disease. *Mol Neurobiol.* (2018) 55:8038–50. doi: 10.1007/s12035-018-0950-y
30. London WB, Castleberry RP, Matthay KK, Look AT, Seeger RC, Shimada H, et al. Evidence for an age cutoff greater than 365 days for neuroblastoma risk group stratification in the Children's Oncology Group. *J Clin Oncol.* (2005) 23:6459–65. doi: 10.1200/JCO.2005.05.571
31. Maris JM. Recent advances in neuroblastoma. *N Engl J Med.* (2010) 362:2202–11. doi: 10.1056/NEJMra0804577
32. Strother DR, London WB, Schmidt ML, Brodeur GM, Shimada H, Thorner P, et al. Outcome after surgery alone or with restricted use of chemotherapy for patients with low-risk neuroblastoma: results of Children's Oncology Group study P9641. *J Clin Oncol.* (2012) 30:1842–8. doi: 10.1200/JCO.2011.37.9990
33. Baker DL, Schmidt ML, Cohn SL, Maris JM, London WB, Buxton A, et al. Outcome after reduced chemotherapy for intermediate-risk neuroblastoma. *N Engl J Med.* (2010) 363:1313–23. doi: 10.1056/NEJMoa1001527
34. Lee EJ, Tournier C. The requirement of uncoordinated 51-like kinase 1 (ULK1) and ULK2 in the regulation of autophagy. *Autophagy.* (2011) 7:689–95. doi: 10.4161/auto.7.7.15450
35. Wang B, Iyengar R, Li-Harms X, Joo JH, Wright C, Lavado A, et al. The autophagy-inducing kinases, ULK1 and ULK2, regulate axon guidance in the developing mouse forebrain via a noncanonical pathway. *Autophagy.* (2018) 14:796–811. doi: 10.1080/15548627.2017.1386820
36. Juricek L, Coumoul X. The aryl hydrocarbon receptor and the nervous system. *Int J Mol Sci.* (2018) 19:2504. doi: 10.3390/ijms19092504
37. Grunwald DS, Otto NM, Park JM, Song D, Kim DH. GABARAPs and LC3s have opposite roles in regulating ULK1 for autophagy induction. *Autophagy.* (2020) 16:600–14. doi: 10.1080/15548627.2019.1632620
38. Zhang Y, Wang F, Han L, Wu Y, Li S, Yang X, et al. GABARAPL1 negatively regulates Wnt/β-catenin signaling by mediating Dvl2 degradation through the autophagy pathway. *Cell Physiol Biochem.* (2011) 27:503–12. doi: 10.1159/000329952
39. Poillet-Perez L, Jacquet M, Hervouet E, Gauthier T, Fraichard A, Borg C, et al. GABARAPL1 tumor suppressive function is independent of its conjugation to autophagosomes in MCF-7 breast cancer cells. *Oncotarget.* (2017) 8:55998–6020. doi: 10.18632/oncotarget.19639

40. Zhang Y, Li G. A tumor suppressor DLC1: the functions and signal pathways. *J Cell Physiol.* (2020) 235:4999–5007. doi: 10.1002/jcp.29402
41. Maruyama T, Noda NN. Autophagy-regulating protease Atg4: structure, function, regulation and inhibition. *J Antibiotics.* (2017) 71:72–8. doi: 10.1038/ja.2017.104
42. Jin J, Hu K, Ye M, Wu D, He Q. Rapamycin reduces podocyte apoptosis and is involved in autophagy and mTOR/P70S6K/4EBP1 signaling. *Cell Physiol Biochem.* (2018) 48:765–72. doi: 10.1159/000491905
43. Berliocchi L, Chiappini C, Adornetto A, Gentile D, Cerri S, Russo R, et al. Early LC3 lipidation induced by d-limonene does not rely on mTOR inhibition, ERK activation and ROS production and it is associated with reduced clonogenic capacity of SH-SY5Y neuroblastoma cells. *Phytomedicine.* (2018) 40:98–105. doi: 10.1016/j.phymed.2018.01.005
44. Ji C, Zhao H, Li D, Sun H, Hao J, Chen R, et al. Role of Wdr45b in maintaining neural autophagy and cognitive function. *Autophagy.* (2020) 16:615–25. doi: 10.1080/15548627.2019.1632621
45. Sasaki T, Lian S, Khan A, Llop JR, Samuelson AV, Chen W, et al. Autolysosome biogenesis and developmental senescence are regulated by both Spns1 and v-ATPase. *Autophagy.* (2017) 13:386–403. doi: 10.1080/15548627.2016.1256934
46. He P, Peng Z, Luo Y, Wang L, Yu P, Deng W, et al. High-throughput functional screening for autophagy-related genes and identification of TM9SF1 as an autophagosome-inducing gene. *Autophagy.* (2009) 5:52–60. doi: 10.4161/auto.5.1.7247
47. Ma L, Bajic VB, Zhang Z. On the classification of long non-coding RNAs. *RNA Biol.* (2013) 10:925–33. doi: 10.4161/rna.24604
48. Bhan A, Soleimani M, Mandal SS. Long noncoding RNA and cancer: a new paradigm. *Cancer Res.* (2017) 77:3965–81. doi: 10.1158/0008-5472.CAN-16-2634
49. Tang Y, Cheung BB, Atmadibrata B, Marshall GM, Dinger ME, Liu PY, et al. The regulatory role of long noncoding RNAs in cancer. *Cancer Lett.* (2017) 391:12–9. doi: 10.1016/j.canlet.2017.01.010
50. Russell MR, Penikis A, Oldridge DA, Alvarez-Dominguez JR, McDaniel L, Diamond M, et al. CASC15-S is a tumor suppressor lncRNA at the 6p22 neuroblastoma susceptibility locus. *Cancer Res.* (2015) 75:3155–66. doi: 10.1158/0008-5472.CAN-14-3613
51. Li D, Wang X, Mei H, Fang E, Ye L, Song H, et al. Long noncoding RNA pancEts-1 promotes neuroblastoma progression through hnRNPK-mediated β-catenin stabilization. *Cancer Res.* (2018) 78:1169–83. doi: 10.1158/0008-5472.CAN-17-2295
52. Liu PY, Tee AE, Milazzo G, Hannan KM, Maag J, Mondal S, et al. The long noncoding RNA lncNB1 promotes tumorigenesis by interacting with ribosomal protein RPL35. *Nat Commun.* (2019) 10:5026. doi: 10.1038/s41467-019-12971-3
53. Yan K, Tian J, Shi W, Xia H, Zhu Y. LncRNA SNHG6 is associated with poor prognosis of gastric cancer and promotes cell proliferation and EMT through epigenetically silencing p27 and sponging miR-101-3p. *Cell Physiol Biochem.* (2017) 42:999–1012. doi: 10.1159/000478682
54. Liang R, Xiao G, Wang M, Li X, Li Y, Hui Z, et al. SNHG6 functions as a competing endogenous RNA to regulate E2F7 expression by sponging miR-26a-5p in lung adenocarcinoma. *Biomed Pharmacother.* (2018) 107:1434–46. doi: 10.1016/j.biopha.2018.08.099
55. Xu M, Chen X, Lin K, Zeng K, Liu X, Xu X, et al. lncRNA SNHG6 regulates EZH2 expression by sponging miR-26a/b and miR-214 in colorectal cancer. *J Hematol Oncol.* (2019) 12:3. doi: 10.1186/s13045-018-0690-5
56. Hu Y, Ma Y, Liu J, Cai Y, Zhang M, Fang X. LINC01128 expedites cervical cancer progression by regulating miR-383-5p/SFN axis. *BMC Cancer.* (2019) 19:1157. doi: 10.1186/s12885-019-6326-5

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Meng, Li, Fang, Feng and Zhao. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Oncology

REVIEW
published: 12 November 2020
doi: 10.3389/fonc.2020.597743

Multifaceted WNT Signaling at the Crossroads Between Epithelial-Mesenchymal Transition and Autophagy in Glioblastoma

Bárbara Paranhos Coelho†, *Camila Felix de Lima Fernandes*†, *Jacqueline Marcia Boccacino, Maria Clara da Silva Souza, Maria Isabel Melo-Escobar, Rodrigo Nunes Alves, Mariana Brandão Prado, Rebeca Piatniczka Iglesia, Giovanni Cangiano, Giulia La Rocca Mazzaro and Marilene Hohmuth Lopes**

Laboratory of Neurobiology and Stem Cells, Institute of Biomedical Sciences, Department of Cell and Developmental Biology, University of São Paulo, São Paulo, Brazil

OPEN ACCESS

Edited by:
Eugenia Morselli, Pontificia Universidad Católica de Chile, Chile

Reviewed by:
Fabrizio Marcucci, University of Milan, Italy
Sabrina Di Bartolomeo, University of Molise, Italy

***Correspondence:**
Marilene Hohmuth Lopes
marilenehl@usp.br

†*These authors have contributed equally to this work*

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *21 August 2020*
Accepted: *19 October 2020*
Published: *12 November 2020*

Citation:
Coelho BP, Fernandes CFL, Boccacino JM, Souza MCS, Melo-Escobar MI, Alves RN, Prado MB, Iglesia RP, Cangiano G, Mazzaro GLR and Lopes MH (2020) Multifaceted WNT Signaling at the Crossroads Between Epithelial-Mesenchymal Transition and Autophagy in Glioblastoma. Front. Oncol. 10:597743. doi: 10.3389/fonc.2020.597743

Tumor cells can employ epithelial-mesenchymal transition (EMT) or autophagy in reaction to microenvironmental stress. Importantly, EMT and autophagy negatively regulate each other, are able to interconvert, and both have been shown to contribute to drug-resistance in glioblastoma (GBM). EMT has been considered one of the mechanisms that confer invasive properties to GBM cells. Autophagy, on the other hand, may show dual roles as either a GBM-promoter or GBM-suppressor, depending on microenvironmental cues. The Wingless (WNT) signaling pathway regulates a plethora of developmental and biological processes such as cellular proliferation, adhesion and motility. As such, GBM demonstrates deregulation of WNT signaling in favor of tumor initiation, proliferation and invasion. In EMT, WNT signaling promotes induction and stabilization of different EMT activators. WNT activity also represses autophagy, while nutrient deprivation induces β-catenin degradation *via* autophagic machinery. Due to the importance of the WNT pathway to GBM, and the role of WNT signaling in EMT and autophagy, in this review we highlight the effects of the WNT signaling in the regulation of both processes in GBM, and discuss how the crosstalk between EMT and autophagy may ultimately affect tumor biology.

Keywords: glioblastoma, autophagy, microautophagy, chaperone-mediated autophagy, epithelial-mesenchymal transition, metabolic reprograming, WNT signaling

INTRODUCTION

Autophagy and epithelial-mesenchymal transition (EMT) are cellular processes that present an intricate correlation, being associated with the development and progression of different tumors. Particularly, autophagy is an essential process for cell survival in healthy tissues and in several types of cancer. Autophagy allows tumor cells to obtain energy from their own cellular structures, consequently turning cell components into possible energy and nutrient reserves, preserving metabolic rates (1–3). It directly contributes to resistance to stress in the tumor microenvironment that is associated with hypoxia, nutritional deficit, acidosis or genotoxicity (3–6). Additionally, autophagy is able to interconvert with EMT, that, in turn, is a cellular process in which the epithelial cells lose their cell-cell junction proteins, apical-basal polarity, and interaction with the basement membrane to acquire mesenchymal cell characteristics. This leads to functional changes and increased migratory capacity with invasive properties (7, 8). The crosstalk between autophagy and EMT, as well as all different molecular pathways involved in these processes, is of great interest in physiological and pathological contexts, especially in cancer.

In tumor cells, EMT is activated in metastasis and invasion, which allows cells to detach from the basement membrane and connect with other cells, extravasating from its initial location and invading adjacent tissues (6, 9). However, in some types of cancer, such as glioblastoma (GBM), EMT appears to be favored by downregulation of autophagic processes (10), and a better comprehension of this entangled regulation might help to clarify GBM biology.

GBM is the most common and malignant glioma, classified as a grade IV brain tumor derived from glial cells (11). Besides high mitotic activity, microvascular proliferation, necrosis, cellular polymorphism, and substantial infiltrative capacity, this tumor is highly heterogeneous and is mainly characterized by resistance to treatment (12, 13). The standard treatment for newly diagnosed tumors is surgical resection with radiation, followed by chemotherapy with temozolomide (TMZ) (13). Along with radiotherapy and TMZ, bevacizumab, a monoclonal antibody against vascular endothelial growth factor (VEGF), and lomustine, an alkylating agent, are current options for recurrent GBM (14). However, a high recurrence rate and failure to respond to therapy lead patients to an average survival time of 15 months (15, 16). A subpopulation of GBM cells with stem-like features termed glioblastoma stem cells (GSCs) is characterized by self-renewal and differentiation into distinct neural cell types, which greatly contributes to the intratumor heterogeneity of GBM (17). GSCs are associated with GBM maintenance, progression and resistance to therapy, contributing to the highly aggressive phenotype (18, 19).

Several signaling pathways upregulated in GBM are involved in cell survival, growth and invasiveness that sustain tumor development and confer resistance to therapy and to harsh microenvironments (20). Among these pathways, current literature suggests that aberrant activation of the Wingless (WNT) signaling contributes to GBM pathology through different cell processes, such as proliferation (21, 22), motility (23–25), cell fate specification (26), and maintenance of stemness properties (27, 28). Interestingly, WNT signaling pathway emerged as a pivotal player to mediate the crosstalk between autophagy and EMT, regulating molecules and connecting to other important signal transduction cascades, such as the mTOR signaling pathway.

Mammalian target of rapamycin (mTOR) signaling pathway is also upregulated in GBM (29). mTOR can establish two distinct multiprotein complexes: mTORC1, that promotes its effects downstream of PI3K/AKT signaling, regulating a diverse variety of cellular processes such as glucose, lipid and nucleotide metabolism, protein biosynthesis and degradation (30); and mTORC2, responsible for phosphorylation of AKT on Ser473, which stimulates its maximal activation (31). In GBM, the PTEN/PI3K/AKT/mTOR pathway is one of the most deregulated, contributing to tumor development and progression (32). Furthermore, mTOR is highly associated with WNT signaling (33), and both WNT pathway and mTOR complexes are involved with autophagy and EMT in GBM, as will be discussed throughout this review.

Thus, we analyze the current knowledge on the role of WNT signaling in autophagy and EMT in the context of GBM, discussing the existent crosstalk between these processes. Notably, we consider how autophagy, EMT and WNT signaling may be interconnected to promote GBM development, progression and therapy resistance.

WNT SIGNALING PATHWAY AND ITS ROLE IN GLIOBLASTOMA

WNTs are secreted glycoproteins rich in cysteine and composed of 300–400 amino acids (34). WNT signaling is classified as the canonical or β-catenin-dependent pathway, and the non-canonical or β-catenin-independent pathway.

In the canonical WNT signaling (**Figure 1**), WNT proteins (WNT1, WNT3A, WNT7A) bind to the membrane receptor complex of Frizzled (FZD) and low-density lipoprotein receptor-related protein 5/6 (LRP5/6), recruiting Dishevelled (DVL). This ultimately leads to stabilization and translocation of the transcription factor β-catenin to the nucleus, where it activates WNT target genes, such as *AXIN2*, *C-MYC* and *CCND1* (cyclin D1), through binding with the complementary transcription factors T-cell factor (TCF) and the lymphoid enhancer factor (LEF) (**Figure 1**) (34). In the lack of WNT binding, β-catenin is phosphorylated by casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3), which leads to the rapid degradation of β-catenin by the proteasome through a destruction complex with involvement of axin-1 and adenomatous polyposis coli (APC) (34). The existence of a low concentration of β-catenin in the nucleus induces the formation of the transcriptional co-repressor Groucho-TCF/LEF complex, which downregulates the expression of WNT target genes (35).

The non-canonical pathway is further divided into two processes: the planar cell polarity (PCP) and the WNT/Ca^{2+} cascade pathways (**Figure 1**). In the first, activation of the PCP pathway leads to determination of the polarity of cells, in

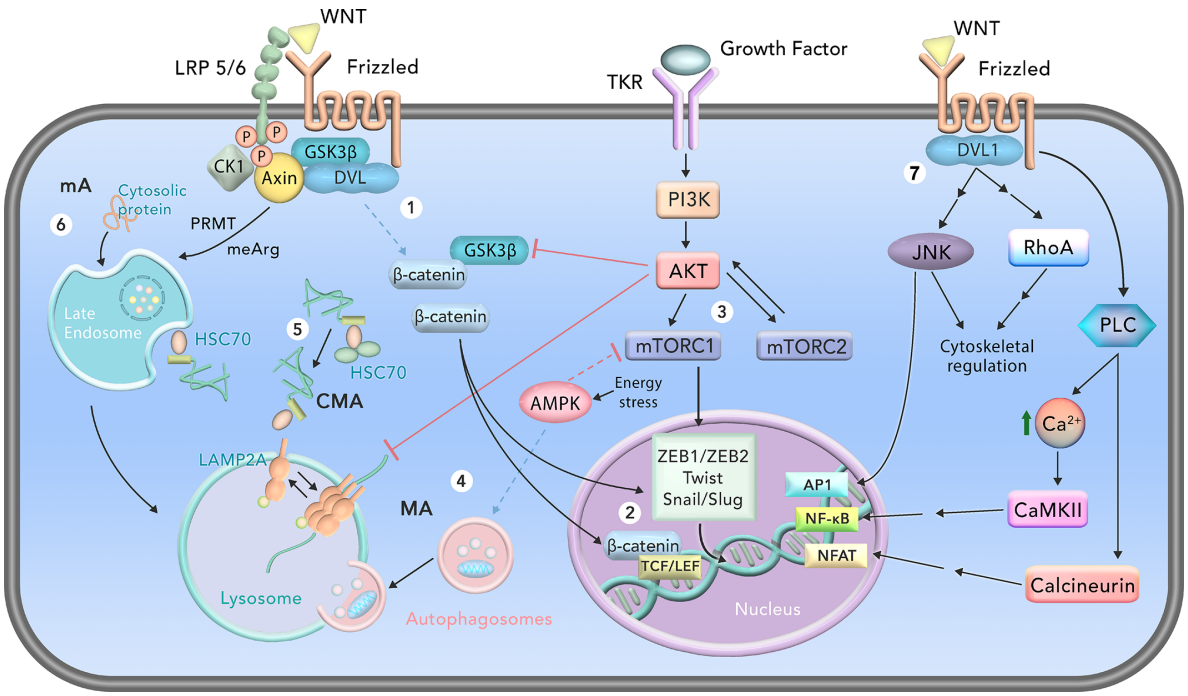


FIGURE 1 | Schematic representation of cell signaling pathways involved in autophagy and epithelial-mesenchymal-transition (EMT). 1) In the canonical WNT signaling, β-catenin stabilizes and translocates to the nucleus after binding of WNT to Frizzled and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) receptors. GSK3 is sequestered together with proteins of the destruction complex of β-catenin (DVL, axin, CK1). 2) In the nucleus, β-catenin binds to TCF/LEF transcriptional factors, activating its target genes. β-catenin also stimulates transcription of important EMT transcriptional factors such as ZEB1/ZEB2, Twist and Snail/Slug. 3) EMT is also induced by the mammalian Target of Rapamycin Complex 1 (mTORC1) signaling, through activation of the transcriptional factors ZEB1/ZEB2, Twist and Snail/Slug. mTORC1 and mTORC2 are activated by tyrosine kinase receptor (TKR) signaling through PI3K/AKT. mTORC2 also stimulates AKT. 4) Depending on the energy status of the cell, activation of AMP Kinase (AMPK) inhibits mTORC1 and induces macroautophagy (MA). 5) Chaperone-mediated autophagy (CMA) degrades cytosolic proteins that possess the KFERQ or KFERQ-like motif through recognition and binding of the heat shock-cognate chaperone of 71 kDa (HSC70) and a cochaperone complex. HSC70 targets the substrate protein to the lysosomal membrane, where it binds monomeric lysosome-associated membrane protein type 2A (LAMP2A) and induces its multimerization and stabilization. The substrate protein is unfolded and translocated into the lysosome for degradation through multimerized LAMP2A. 6) In mammals, microautophagy (mA) occurs in the late endosome, in a process called endosomal microautophagy. It can degrade cytosolic proteins, and some are recognized by HSC70. Some proteins, such as GSK3, are targeted for mA through arginine methylation (meArg) by protein arginine methyltransferases (PRMTs). After entering the late endosome, this organelle fuses with lysosomes to complete the mA cycle. 7) In the noncanonical WNT signaling, WNTs bind to Frizzled receptors and DVL1 is recruited to the membrane. c-Jun N-terminal kinases (JNKs), RhoA and phosphoinositide phospholipase C (PLC) can be activated by noncanonical WNT. JNK and RhoA further regulate the cytoskeleton, and JNK induces gene transcription through activator protein 1 (AP1). PLC increases cytosolic Ca^{2+} levels, leading to activation of Ca^{2+}/calmodulin-dependent protein kinase II (CAM-KII) and calcineurin, which activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and nuclear factor of activated T cells (NFAT), respectively. Solid black lines represent activation, while solid red lines represent inhibition. Double black lines represent indirect activation. Dashed lines indicate protein interactions when the WNT/β-catenin and mTOR signaling are not activated.

addition to affecting cellular shape and migration. In the PCP pathway, WNT ligands (WNT4, WNT5A and WNT11) bind to the FZD receptor and to one of two co-receptors: receptor-like tyrosine kinase (RYK) or receptor tyrosine kinase-like orphan receptor (ROR). This binding induces DVL, which activates DVL-associated activator of morphogenesis 1 (DAAM1), profilin, and the protein Rho- and RAS-related C3 botulinum toxin substrate 1 (RAC1). DAAM1 then activates the RAS homolog family member A (RhoA), which regulates the cytoskeleton through the Rho-associated kinase (ROCK). RAC1 induces c-Jun terminal kinases (JNKs), which directly or indirectly activate cytoskeletal alterations (20, 35). Secondly, the WNT/Ca^{2+} pathway is important during embryogenesis, in the formation of the embryonal dorsal axis, gastrulation and tissue morphogenesis. WNT ligands bind to the FZD receptor, which promotes activation of DVL1 and a G-protein. This complex results in the intracellular release of Ca^{2+}, which activates the Ca^{2+}/calmodulin-dependent protein kinase II (CAM-KII) and calcineurin, along with two transcription factors: nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and nuclear factor of activated T cells (NFAT). Additionally, certain WNT ligands of the non-canonical pathways can inhibit the canonical WNT pathway (20).

Moreover, certain extracellular antagonists can control WNT signaling, by extracellularly interacting with WNT ligands or receptors, preventing the proper binding of WNT proteins and impairing the maturation of receptors (36). For instance, Dickkopf (DKK1, DKK2, DKK3, DKK4, and DKKL1) is a family of secreted

glycoproteins that modulate WNT signaling mainly by binding to LRP5/6, preventing the triggering of signaling (37). These features of DKK proteins are being explored due to its role in different types of cancers (38, 39). Additionally, other antagonists such as WNT inhibitor factor 1 (WIF1), frizzled-related protein family (FRP) – also referred as secreted frizzled-related proteins (sFRP) – and Cerberus work in similar ways (40).

Growing evidence has established a critical role of WNT signaling pivotal players during the onset of GBM. Several of the aforementioned molecules can be associated with the aberrant activation of WNT pathway in GBM, and a particular interest has been attributed to GSCs. WNT5A is epigenetically activated in GSCs due to absence of the H3K27me3 repressive mark (41). Another important molecule is LEF1, and its downregulation inhibits the capacity of self-renewal and expression of stemness markers, such as CD133 and Nestin (42), which negatively impacts proliferation, migration and *in vitro* invasion of GBM cells, playing a role in the EMT process (43). Additionally, the RYK receptor is upregulated in GSCs of patient samples, activating WNT pathway, promoting stemness and improving cell motility (44). Moreover, one of the molecules responsible for translocating and stabilizing β-catenin in GBM is forkhead box protein M1 (FOXM1), which may induce the canonical pathway independently from ligand binding (45). This factor can also be linked to the SOX2 promoter, a classic marker of the stem cell phenotype, known for promoting clonogenic growth in GBM (46).

Using a whole-genome approach, Foltz et al. (47) found that DKK1, sFRP1 and WIF1 were epigenetically silenced in GBM cells (47). The FRP genes are frequently found hypermethylated and inhibited during tumor development. Demethylation of the FRP gene promoter in human glioma cell lines led to an increase in phosphorylated β-catenin in the cytosol, attenuating tumorigenesis (48). Expression of FRPs promotes apoptosis through a possible activation of the DNA damage machinery through FAS-p53, activating the non-canonical WNT/Ca^{2+} pathway and the release of reactive oxygen species (ROS) (49). FRP4 treatment, in conjunction with TMZ, inhibited the canonical WNT pathway and was associated with a decrease in the expression of mesenchymal markers such as N-cadherin, Twist and Snail, along with a greater expression of epithelial markers, such as E-cadherin, showing the role of the inhibitor in reversing EMT (49). In addition, FRP4 chemically sensitizes GSCs, which decreases stemness properties that contribute to therapeutic resistance (49).

WIF1 has a negative influence on the ability of tumor cells to invade and migrate *in vitro* and *in vivo* (24). This suppressor phenotype is due to the downregulation of the canonical and non-canonical WNT pathways, with the inhibition of β-catenin-independent pathway being mediated by the sequestration of WNT5A, a ligand overexpressed in GBM. This inhibition results in decreased phosphorylation of p38-MAPK, reduction of intracellular Ca^{2+} concentration, and reduction in the expression of the metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), a long non-coding RNA and a key invasion regulator (24). Moreover, recovering the expression of DKK1 in GBM cells results in inhibition of the WNT pathway, leading to growth suppression and decreased colony formation (47).

WNT signaling is also fundamental in the crosstalk between microglia and GBM (50). WNT3A derived from GBM induces upregulation of stress-inducible protein 1 (STI1/HOP), interleukin 10 (IL-10), and arginase-1 (ARG-1) in tumor-associated microglia. Together, these molecules collaborate with tumor immune evasion and increase in cell proliferation and migration (51–53). Another report showed that WNT3A increases the level of expression of programmed death-ligand 1 (PD-L1) on the surface of GBM cells, which is an important protein that inhibits the activation, expansion and effector functions of cells with antitumor activity (T^{CD8+}) (54), corroborating the importance of WNT3A in immune evasion (55). Furthermore, WNT3A also acts on the WNT/β-catenin/FRA1 axis, a cascade that plays a crucial role in the aggressiveness of glioma and in EMT activation (56).

Due to GBM heterogeneity, EMT is usually incomplete, with associated transient phenotypic states, which results in a combination of epithelial and mesenchymal characteristics (9). It is noteworthy that the infiltrative peripheral region of GBM contains cells with mesenchymal characteristics, with higher WNT/β-catenin pathway activation (5), conferring a specific type of EMT-like process in GBM. As will be further discussed in this review, EMT and autophagy are influenced by WNT signaling, and the crosstalk between these pathways plays an important role in GBM pathology.

AUTOPHAGY IN THE CONTEXT OF GLIOBLASTOMA AND WNT SIGNALING

Autophagy is a greatly conserved process in eukaryotes, and it has an important role in homeostasis and cell components renewal in the face of adverse conditions. Autophagy degrades defectively folded proteins and dysfunctional organelles, such as mitochondria and peroxisomes, in addition to being essential for cell survival under microenvironmental stress (57).

Mammalian autophagy is classified in three types: microautophagy (mA), chaperone-mediated autophagy (CMA), and macroautophagy (MA) (57, 58). All three types can coexist within a cell and, despite all having the same overall purpose of cargo recycling or degradation of cargo in the lysosome, each one has its own delivery mechanisms and regulation. Remarkably, the modulation of different autophagy types has been associated to GBM biology and will be discussed as follows.

Microautophagy in Glioblastoma

In mammals, mA ensues through different mechanisms that share some similarities. It can occur directly in lysosomes, in which they extend and wrap themselves around a portion of the cytoplasm, or they form arm- or flap-like protrusions that surround parts of the cytoplasm (59, 60). However, mammalian mA is mostly described as endosomal microautophagy (eMI), occurring in late endosomes/multivesicular bodies (MVBs) (61), which then fuse with lysosomes for breakdown of their cargo (**Figure 1**). This process is attributed to bulk degradation of proteins and other cytosolic components

engulfed by late endosomes and can also selectively degrade specific proteins with the assistance of chaperones such as heat shock cognate 71 kDa (HSC70) (61). Interestingly, Sato et al. (62) demonstrated that rapamycin induces eMI, independently of MA, while lacking effect upon CMA (62). This activation is triggered by the nuclear transcription factor EB (TFEB) which increases the expression of several genes related to mA (62).

Recent studies have shown a connection between the WNT pathway and mA. WNT activation leads to the sequestration of GSK3 from the cytosol into late endosomes/MVBs through mA (63). WNT signaling also stabilizes other proteins and GSK3 substrates besides β-catenin. Moreover, WNT activation is highest in the G2/M phase of the cell cycle and the stabilization of GSK3 substrates is thought to decrease the rate of GSK3-dependent protein degradation to prepare for cell division. This shifts the molecules from proteasome degradation (which consumes a large amount of ATP) to microautophagic degradation, which depends on hydrolases inside lysosomes and does not require ATP consumption (64). Despite the research gathered about WNT and mA, there is still an extensive gap in the knowledge of the intricate regulatory mechanisms and the physiological function attributed to mA/eMI in mammals.

eMI is suggested to control protein quality (65) by limiting the intracellular levels of key proteins, working as a regulator of specific cellular processes (66, 67). Other than its physiological roles, mA may be involved with cancer growth. Albrecht et al. (64) showed, for example, that arginine methylation (meArg), promoted by protein arginine methyltransferases (PRMTs), is important for mA, being necessary for the WNT-induced GSK3 sequestration and subsequent microautophagic degradation (64). GSK3 and the tumor suppressor SMAD4 are methylated and targeted to late endosomes/MVBs upon WNT signaling (**Figure 1**), suggesting that inhibition of PRMT1 might oppose cancer progression. Additionally, axin has also been shown as a PRMT1 substrate. As mentioned above, axin acts as a scaffold protein and plays a major role in the destruction complex of β-catenin (64). Upon WNT activation, axin is recruited to the plasma membrane, where it binds to the WNT receptor complex. Arginine methylation in axin has been shown to increase its binding to GSK3, and the WNT coreceptors and GSK3 are then translocated together with axin to MVBs (64).

Thus, meArg has been associated with cancer progression and PRMTs have been considered as novel drug targets (68). In GBM, PRMT2 was shown to be overexpressed and is correlated with a poor prognosis (69). PRMT5 is also highly expressed in GBM, promotes self-renewal of GBM neurospheres (70), and resistance to mTOR inhibition in GBM cells lines and short-term patient cultures (71). mA, however, has not been investigated in GBM. Due to the recent discoveries on the requirement of meArg and mA to WNT signaling activation, this autophagic process might be involved with GBM biology and merits further investigation.

Chaperone-Mediated Autophagy in Glioblastoma

CMA contributes to the control of cellular quality and maintenance of the proteome (72, 73). Exposure of the KFERQ-like domain of proteins targets them to CMA and selective eIM. In CMA, exposure of this motif leads to its recognition by the chaperone HSC70, and consequent binding of this chaperone to the substrate protein, followed by targeting of this complex directly to the lysosomal membrane (74). This mechanism differs from mA and MA, in which the cargo is sequestered into vesicles before being directed to the lysosome (75). Interestingly, selective blockage of CMA induces activation of MA; however, MA cannot compensate for CMA functions under specific stress conditions, in which CMA plays an essential role (76).

Upon binding of HSC70 to the substrate protein, a chaperone-cochaperone complex is formed and assists HSC70 to redirect client proteins captured in the cytosol to the lysosome membrane (**Figure 1**) (77). On the lysosome membrane, the chaperone complex and its client proteins bind to the lysosome-associated membrane protein type 2A (LAMP2A), inducing LAMP2A multimerization (78). After unfolding, the substrate is then translocated into the lysosome, where the protein is rapidly degraded by hydrolases (77, 78). It is clear that CMA activity is limited by the levels of LAMP2A on the lysosomal membrane, which makes LAMP2A the rate-limiting component for CMA (79, 80).

In addition to LAMP2A levels, further regulation of CMA occurs at the lysosomal membrane. Glial fibrillary acidic protein (GFAP) and elongation factor 1α (EF1α) regulate the stability of multimeric LAMP2A (81). Lysosomal GFAP, when phosphorylated by AKT1 activated by mTORC2, remains bound to EF1α and it is unable to stabilize multimeric LAMP2A (81). Ultimately, this leads to CMA inhibition regulated by mTORC2. When CMA is necessary, the GTPase RAC1 recruits and stabilizes the phosphatase pleckstrin homology domain and leucine-rich repeat protein phosphatase 1 (PHLPP1) at the lysosomal membrane, and this phosphatase dephosphorylates AKT1. This increases non-phosphorylated GFAP and favors the formation of the LAMP2A multimeric complex (73). Thus, it is possible to hypothesize that CMA inhibition might be regulated by mTORC2 and by WNT signaling, since RAC1 is one of the effectors of the PCP pathway. Moreover, signaling of the retinoic acid receptor α (RARα), associated to differentiation of non-tumor and tumor cells, inhibits transcription of genes required for CMA, highlighting an additional pathway of regulation for this autophagic process in health and, particularly, in cancers such as GBM, in which GSCs are a prominent factor (82).

CMA activity is markedly increased in cancer cells. Noteworthy, an anti- or pro-cancer function of CMA appears to depend, at least in some extent, on the status of cell transformation (83) and stage of the specific tumor (84, 85). This facet supports the importance of a context-dependent therapy investigation, requiring specialized research and a more translational approach.

There are very few studies that specifically explore the function of CMA in GBM, but the aggressiveness and high heterogeneity of this tumor raise important questions about the extent to which CMA may be contributing to these features. Maititi et al. (86) reported that the treatment of different GBM cell lines with curcumin and solid lipid curcumin particles downregulated LAMP2A levels, consequently decreasing CMA (86). Alternatively, regarding CMA activation, TMZ-resistant and

TMZ-sensitive cells respond differently, according to hypoxia-inducible factor 1α (HIF-1α) expression and their basal levels of cytoplasmic ROS. CMA was shown to be the main process through which TMZ inhibits activity of HIF-1α in sensitive cells, promoting apoptosis and increasing responsiveness to the drug (87). Downregulation of LAMP2A blocks HIF-1α degradation through CMA and is sufficient to induce a TMZ-resistant phenotype in GBM sensitive cells, reducing the expression of several apoptosis-related genes upon treatment (87). Further research showed CMA is activated following cytoplasmic release of mitochondrial ROS, since concomitant increase in LAMP2A and HSC70 is observed with induction of oxidative stress. In accordance, inhibition of mitochondrial ROS downregulates LAMP2A and HSC70 (88). TMZ-sensitive cells present low basal levels of ROS, which in turn are increased with TMZ treatment, consequently activating CMA and pro-apoptotic genes. In contrast, TMZ-resistant cells have high basal levels of ROS and fail to activate CMA upon TMZ treatment (88). Remarkably, knockout of LAMP2A leads to CMA blockage, which is sufficient to trigger the switch of sensitive to resistant responsiveness (88). In T cells, production of mitochondrial ROS by engagement of the T cell receptor induces nuclear translocation of NFAT, which binds to the *LAMP2* promoter and activates CMA (89). As discussed in section 2, the non-canonical β-catenin-independent WNT/Ca^{2+} cascade activates NFAT (90). Although non-canonical WNT pathways are usually related to embryonic patterning and morphogenesis, their aberrant regulation increases invasiveness in GBM (20). It is interesting to interrogate whether non-canonical WNT signaling through NFAT could be involved with activation of CMA in GBM cells, since ROS can be involved with the activation of both NFAT and CMA.

Another compelling mechanism is the ability of GBM cells to induce CMA in pericytes that surround the tumor, suppressing antitumor response and facilitating tumor progression. Specifically, interaction between GBM cells and pericytes of the peritumoral area increases ROS and subsequently LAMP2A levels, inducing nanotube formation and downregulation of the secretion of vesicles containing cytotoxic molecules (91). This results in promotion of direct interaction between these cells and abrogates inflammatory response (91). Supporting this, high levels of granulocyte-macrophage colony stimulating factor (GMCSF), molecule that promotes immunosuppression, were found in GBM cells after interaction with pericytes (91).

A thought-provoking aspect of CMA contribution to GBM is that chaperones and cochaperones that function in CMA play important roles in GBM biology (92). STI1/HOP, a known component of the CMA translocation complex (77), is upregulated in GBM cells. High levels of both STI1/HOP and its partner cellular prion protein (PrP^C) are correlated with increased proliferation of GBM cells (93). Moreover, interaction between STI1/HOP-PrP^C was found to sustain proliferation of GBM stem-like cells and tumor growth *in vivo*, contributing to its aggressiveness (94). Curiously, a mutant form of PrP^C interacts with LAMP2A and HSC70, raising the question whether the non-mutated form of PrP^C can also interact with CMA-related proteins (95). Interestingly, WNT3A increases STI1/HOP expression in GBM cells (53), suggesting that WNT signaling could regulate CMA, or that GBM cells take advantage of increased STI1/HOP expression to sustain tumor growth and modulate GSCs. Furthermore, since mA favors WNT signaling through GSK3 degradation, these data demonstrate how WNT signaling appears closely involved with autophagic processes, both by depending on them to be induced and by participating in their indirect activation.

In addition to STI1/HOP, other proteins of the translocation complex are proposed to be related to GBM biology, such as BAG1. BAG1 confers proliferation advantage in GBM cell lines in serum starvation conditions, a CMA-inducing environment, through interaction with BCL-2 (96). Although the mechanisms described for the function of these proteins in the biology of GBM have not yet been directly associated with CMA, it may be an interesting strategy to investigate the contribution of CMA in this context, considering the intricate relationship of its components with the aggressiveness of GBM. Thus, evidence gathered here demonstrate that, despite knowing the main processes and molecules involved in CMA, novel intermediate players that finely tune the mechanism - especially in the context of disorders such as cancer - need to be unraveled to actively consider CMA as a viable, well controlled and safe therapeutic approach.

Macroautophagy in Glioblastoma

MA involves the formation of an isolated vesicle, termed autophagosome, for transportation of the materials to the lysosome (**Figure 1**) (97). It consists of the following critical steps: initiation, nucleation, maturation and degradation of the cargo, with consequent release of the degradation yields to the cytosol (98). In the initiation step, there is the formation of a kinase complex comprised of Unc-51-like kinase 1 and 2 (ULK1/2), family 200-kDa interacting protein (FIP200), the mammalian ATG13 (mATG13); and ATG101 (99). mTORC1 inhibition under starvation activates ULK1/2, triggers phosphorylation of mATG13 and ULK1/2, and induces ULK-dependent phosphorylation of FIP200 (100). Moreover, studies demonstrate AMP-activated protein kinase (AMPK) binds to the ULK1-mTORC1 complex and induces ULK1-mediated autophagy through inhibition of mTORC1 by phosphorylation of Raptor (**Figure 1**) (101, 102).

In the stage of nucleation, the ULK1 complex targets another protein complex, which includes a class III phosphatidylinositol-3 kinase (VPS34), beclin 1, ATG14L, VPS15, and the autophagy beclin 1 regulator 1 (AMBRA-1) (103, 104). Subsequent to phagophore nucleation, in the maturation phase, there is the formation of two protein conjugations. During one of them, the processing of microtubule associated protein 1 light chain 3 (LC3) by ATG4 occurs to yield LC3-I, which in turn is bound to the target lipid phosphatidylethanolamine to generate processed LC3-II (105). LC3-II is integrated in the phagophore where it interacts with adaptor molecules, such as p62/SQSTM1, to select cargo for degradation and to label the phagophore membrane as autophagic (106). Promptly after autophagosome completion, most of the ATG proteins detach from the vesicle (107) triggering its fusion with the lysosome to form the autolysosome (108). In mammalian cells, autophagosome-lysosome fusion is regulated by LAMP2 and the small GTPase RAB7 (109, 110).

The role of MA in cancer and in GBM has been extensively covered in the literature (for more information, refer to (98, 111). Here we will focus on the involvement of the WNT pathway and MA in GBM.

In GBM cell lines, WNT5A/β-catenin signaling was demonstrated to modulate MA (112). The macroautophagic flux was increased upon impairment of WNT/β-catenin signaling *via* the inhibition of the downstream effector TCF (113). Similarly, extracellular DKK1 triggers MA by disrupting the communication of WNT ligands to their cell surface receptors, thus inhibiting WNT cascades (113).

Furthermore, WNT signaling proteins are also targets of MA. The WNT/β-catenin target genes *AXIN2* and *CCND1* become downregulated upon starvation-induced MA or mTOR blockage in GBM, although *C-MYC* expression shows no alteration (114). The same autophagy-inducer mechanisms were shown to decrease the expression of the DVL2 protein (115). This was also observed by Colella et al. (114), where MA led to a decrease in the expression of DVL2 and nuclear β-catenin in GBM cells (114). The study also showed that β-catenin was located in nearby plasma membrane regions and engaged with N-cadherin to form structures similar to epithelial-like cell-cell junctions (114).

The protein Von Hippel-Lindau (VHL) was demonstrated to ubiquitylate DVL2, which leads to its macroautophagic degradation (115). Noteworthy, data from the literature demonstrate that VHL also promotes the ubiquitination of the HIF-1α and 2α (HIF-2α) in GBM (116–119). Interestingly, in renal cell carcinoma, HIF-2α was shown to be degraded *via* both proteasome system and macroautophagic pathway in a VHL-dependent manner. Inhibition of MA led to HIF-2α degradation by the proteasome, whereas suppression of the proteasome caused HIF-2α degradation by MA (120). Furthermore, HIF-1α was demonstrated to induce MA in GBM as a mechanism of resistance to antiangiogenic therapy (121). Evidence indicates an interaction between the WNT/β-catenin pathway and HIF proteins in both physiological and pathological conditions. For instance, the crosstalk between WNT signaling and HIF-1α was demonstrated to be involved in maintenance of GSCs stemness, since their interaction transcriptionally regulates the neuronal differentiation of these cells (122).

In summary, the aforementioned studies point out to the importance of the WNT signaling and MA in GBM biology. As discussed, there is an important crosstalk between WNT/β-catenin signaling, MA and hypoxic pathways that warrants further investigation. The relationship between these pathways indicates the importance of metabolic reprogramming in GBM, in a context where nutrient availability may be scarce, and MA may be downregulated through induction of WNT/β-catenin and PI3K/AKT/mTOR signaling.

EPITHELIAL-MESENCHYMAL TRANSITION IN GLIOBLASTOMA

Epithelial cells can undergo EMT, in which cells lose their apical-basolateral polarity as well as cell-cell adhesion structures in order to obtain an enhanced migratory phenotype (123). Upon transition, the cells become able to degrade the basal membrane using metalloproteases and thus migrate from their original site assisted by cytoskeleton rearrangements (124). As described in the name of the process, the transitioning cell shows increased expression of mesenchymal markers as well as loss of epithelial marker and adhesion molecules (125). Initial onset of EMT is dependent on many proteins that participate in the motility cycle, such as Rho GTPases, RAC1, and CDC42 (126), transcription factors from the SNAI family, such as Snail1 and Slug, as well as homeodomain transcription factors such as ZEB1 and ZEB2 (**Figure 1**) (5, 125). Additionally, many of the above-mentioned proteins - such as Snail1, Slug, ZEB1, ZEB2 and Twist - are able to regulate the switch of expression from E-cadherin to N-cadherin, an important hallmark of EMT (127, 128). This biological phenomenon has a well-known role in wound healing and development (129, 130), in addition to cancer invasion (131). Moreover, EMT is divided into 3 distinct subtypes: type 1 EMT is related to morphogenesis and is modulated by signaling pathways such as WNT/β-catenin, which in turn regulates Snail1 expression (132); type 2 EMT is related to wound healing and fibrosis and can be regulated by Slug, epidermal growth factor (EGF) and transforming growth factor β (TGF-β) pathways (130); and type 3 EMT occurs in cancer cells, playing an important role on tumor invasion and metastasis (133), and can be regulated by cytokines secreted in the tumoral niche such as TGF-β and tumor necrosis factor α (TNFα) (5).

As seen during neurodevelopment, mainly during migration of the neural crest, neuroepithelial cells may undergo transition to a more mesenchymal phenotype and, therefore, increased migratory capacity (134, 135). GBM can co-opt this process to assist cell invasion and migration. Importantly, neural tissues lack E-cadherin expression, although this protein is expressed in GSCs and in a rare subgroup of extremely aggressive GBM cells (5, 136). As GBM cells do not intrinsically express E-cadherin, the switch to N-cadherin, that is classic in EMT, is not entirely observed in this tumor (137). Moreover, distinct motility behaviors observed in GBM were associated to N-cadherin distribution in the cell instead of its expression levels (138). Therefore, GBM cells undergo a process similar to EMT, called EMT-like or glia-to-mesenchymal transition (GMT). Such process can be induced through radiation and be modulated by TGF-β-dependent and TGF-β-independent pathways through mitogen-activated protein kinases (ERK1/2) and GSK3, which in turn modulate Snail expression and localization (139). Likewise, neuronal cells also seem to undergo an EMT-like process, relying on Scratch1 and Scratch2 proteins, members of the Snail superfamily, to repress E-cadherin expression (140).

Additionally, the G protein-coupled receptor LGR5 triggers EMT in GSCs through the WNT/β-catenin cascade (141). Moreover, WNT-C59, an antagonist of the pathway, was able to impair EMT in GSCs (141). The transcription-related protein FRA1 was shown to act downstream of WNT3A signaling, promoting EMT in GBM cells and therefore contributing to the aggressive behavior of this tumor (56). Furthermore, the micro-RNA miR-504 suppresses the expression of the *FZD7* gene, which causes the WNT/β-catenin pathway to be downregulated and affects EMT

(142). Importantly, the transcription factors LEF1 and HOXA13 have both been shown to promote EMT in GBM *via* WNT signaling (143). Furthermore, the WNT/β-catenin cascade was shown to be activated in GBM cells of the mesenchymal subtype, leading to induction of expression of ZEB1, Twist1 and Slug, along with increased migratory capability of these cells (5). These recent findings support the importance of the WNT signaling for induction of EMT in GBM, pointing out to the WNT pathway as a promising therapeutic target for reducing the aggressiveness of this tumor.

CROSSTALK BETWEEN AUTOPHAGY, EPITHELIAL-MESENCHYMAL TRANSITION, AND WNT SIGNALING IN GLIOBLASTOMA

When supporting cancer cells, autophagy can provide nutrients and energy for both EMT and the consequent metastasis, since it can sustain cells for survival in stressful environmental conditions (7). On the other hand, autophagy can also inhibit EMT by modulating the expression of specific proteins. As such, MA can downregulate mTOR signaling, leading to β-catenin and N-cadherin degradation and E-cadherin expression (7, 144). Therefore, it is believed that autophagy is oncosuppressive at initial stages of metastasis, by destabilizing or degrading crucial inducers of EMT, consequently inhibiting this process. If the cell is unable to prevent malignant transformation and metastasis ensues, EMT is activated and autophagy is required for tumor cell survival in environmental stress (5). Additionally, further dimensions should be added to this hypothesis, by considering the role that important pathways, such as the WNT signaling, and the different autophagic processes (mA, CMA, and MA), may have in the balance of oncosuppressive or metastatic function, especially in a highly infiltrative tumor as GBM. In this session, we discuss the evidences for the crosstalk of WNT signaling-autophagy-EMT observed in different aspects of GBM biology (**Figure 2**).

The microenvironment surrounding GBM presents hypoxic niches, which contribute to scarcity of nutrients and to dependence on glycolytic metabolism (145). Hypoxia, and consequent HIF-1α signaling activation, is a known inducer of MA, and also a known EMT promoter in different types of cancers (146–150). In GBM, an intricate relationship of those pathways with WNT signaling can be postulated, since strong correlation in the expression of HIF-1α and β-catenin was demonstrated in tumor cells (151). In addition, HIF-1α promotes the expression of the β-catenin transcriptional partners TCF1 and LEF1, regulating β-catenin nuclear retention (152, 153). This contributes to the activation of target genes associated with GBM tumor invasion and proliferation, such as Snail/Slug and cyclin D1 (154). Remarkably, MA and CMA appear to have opposed roles during GBM responsiveness to hypoxia. As covered in *Chaperone-Mediated Autophagy in Glioblastoma*, CMA decreases HIF-1α activity in GBM cells. On the other hand, hypoxia induction increases MA. Furthermore, the response to curcumin treatment is also opposed: while the treatment induces MA, concomitantly decreasing the expression of β-catenin targets, it also decreases CMA. However, this increased MA by curcumin treatment could be a feedback response of the tumor cell to the CMA inhibition.

Alternatively, HIF-1α/TCF1 activation can also result in neuronal differentiation in GBM cells subjected to hypoxia, and this pathway is primarily activated in the subpopulation of GSCs, being responsible for a pro-differentiation phenotype (122, 155). Interestingly, HIF-1α/TCF4 activation in normoxia triggers transcriptional inhibition of the same regions once activated in hypoxia (122). The differences between the outcomes for tumor biology might rely on the highly heterogenous cell populations in GBM tumors, and the initial spatial-molecular context of these cells. Despite that, different reports advocate hypoxia-mediated pathways working in tumor supporting mechanisms, contributing to chemotherapy resistance and invasion of healthy tissue.

Besides contributing to the distinct above-mentioned mechanisms, HIF-1α is able to bind the hypoxia-response element of *TWIST* proximal promoter and mediate its expression (156). Twist and Snail are targets of β-catenin signaling (156, 157), and are known EMT transcription factors (158), whose levels can be controlled by MA (159). In GBM particularly, a recent report describes the associated increase in MA markers with decreased levels of Twist and Snail in tumor cells treated with a combination of TMZ and curcumin (160). Moreover, Snail and Twist are found increased in GBM, together with other EMT-related transcripts (161). Thus, regulation of the levels of these transcription factors by autophagy represents one of the feedback correlations contributing to the crosstalk between autophagy and EMT, ultimately dictated by WNT upstream signaling.

An additional factor to be considered when exploring the implications of WNT pathway, EMT and autophagy in GBM is FOXM1. FOXM1 is upregulated in GBM (162) and plays a key role in the EMT-like process, directly controlling the expression of the proteins matrix metallopeptidase 2 (MMP2) (163) and A disintegrin and metalloproteinase 17 (ADAM17) (164). WNT signaling works in the stabilization of FOXM1 (165), and FOXM1 is able to directly translocate β-catenin to the nucleus of GBM cells, independently of extracellular ligands (45). Recently, it was shown that FOXM1 binds to the promoter region and controls the overexpression of the ubiquitin-conjugating enzyme E2C (UBE2C), protecting GBM cells from autophagic cell death, probably through PI3K-AKT-mTOR activation (166).

According with this overall increase in WNT activation in GBM, protein expression of the DVL family is also enhanced in this tumor (167–169). Interestingly, autophagic decrease in DVL2 levels impairs β-catenin nuclear localization, concomitant with an epithelial-like phenotype (114). Additionally, the dishevelled-associated antagonist of β-catenin 2 (DACT2) is a DVL-interacting protein that decreases the level of activated β-catenin resulting in suppression of WNT/β-catenin target genes (170). In GBM, decreased levels of DACT2 were proportionally associated

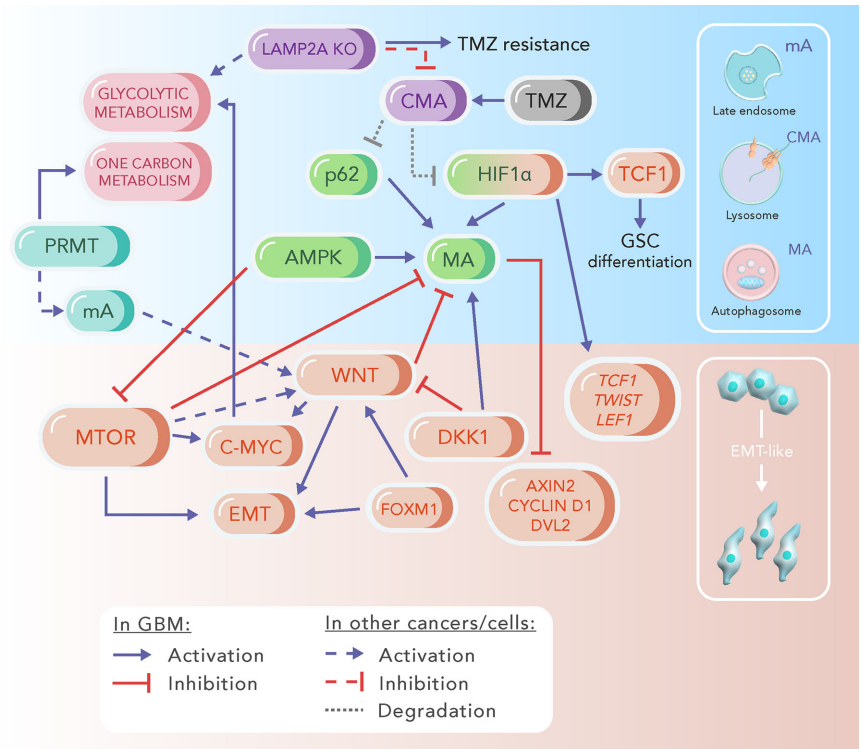


FIGURE 2 | Schematic representation of signaling pathways involved with WNT/β-catenin, autophagy and epithelial-to-mesenchymal transition (EMT) in glioblastoma (GBM). The major players of the highlighted processes are grouped by a colour code, which differentiates microautophagy (mA, light blue), chaperone-mediated autophagy (CMA, purple), macroautophagy (MA, green), EMT (orange) and some metabolic processes (pink). AMPK, AMP Kinase; DKK, Dickkopf; DVL2, dishevelled 2; FOXM1, forkhead box protein M1; HIF1a, hypoxia-inducible factor 1a; LAMP2A KO, knockout of LAMP2A protein; LEF1, lymphoid enhancer factor 1; mTOR, mammalian target of rapamycin; PRMT, protein arginine methyltransferase; TCF1, T-cell factor 1; TMZ, temozolomide.

with shorter patient survival, greater tumor aggressiveness and *in vivo* growth (171), suggesting that an apparent decrease in the levels of this protein favors tumor maintenance. The control of the levels of DACT2 in GBM has not yet been directly associated with autophagy, but the expression of other proteins of the same family were found strongly associated with autophagic processes in cancer cells (172, 173). Those evidences support the fundamental role of the intricate relationship between the EMT-like process, WNT signaling and autophagy for the progression of GBM tumors.

In the context of GBM, angiogenesis provides nutrients for the tumor to grow and favors metastasis (174). Thus, antiangiogenic therapy has been employed for GBM, especially with bevacizumab as adjuvant therapy (175). Although bevacizumab was shown to increase progression-free survival by 6 months, its effects on overall survival are controversial (175). Moreover, bevacizumab treatment generated hypoxia in the tumor microenvironment *in vivo*, and this hypoxia induced by the drug altered the metabolism of GBM cells *in vitro* (176). Interestingly, endostatin is another antiangiogenic drug being considered for GBM therapy. Endostatin is a terminal fragment of collagen XVIII-α 1, binds to α5β1 integrin on endothelial cells and induces autophagy through VPS34, beclin 1 and LC3-II activation (177, 178). Moreover, endostatin was shown to antagonize WNT signaling and to target β-catenin for degradation (179). Endostatin also decreased EMT markers on basal cell carcinoma (180). Additionally, a negative correlation between β-catenin and beclin 1 was reported (181), which could explain the decrease of EMT markers by endostatin, through inhibition of WNT signaling and induction of autophagy. Other reports in the literature confirm a role for WNT signaling in inhibiting autophagy in GBM (182).

Therefore, other therapeutic approaches against GBM are aiming to induce autophagy through mTOR inhibition. The first line of mTOR inhibitors, however, were able to block only mTOCR1 activity (183). As seen throughout this review, mTORC2 is involved with GBM invasion and could inhibit CMA. Moreover, during osteoblast differentiation, WNT3A/LRP5 induces mTORC2/AKT signaling downstream of RAC1 (184). To circumvent this ineffectiveness of initial mTOR inhibitors, novel ATP-competitive mTOR kinase inhibitors (TORKIs) were developed to target both mTOR complexes and are being investigated against GBM (5). Torin 1, for example, induced nutrient deprivation and inhibition of mTOR complexes, resulting in β-catenin re-localization and inhibition of migration (114). Moreover, other molecules that induce autophagy in GBM, such as arsenic trioxide, sodium selenite, and cannabinoids, combined with traditional therapy, were able to increase drug-induced cell death (185–187).

In contrast, autophagy inhibition was also proposed as a therapeutic approach against GBM. TMZ was shown to induce autophagy in GBM cells instead of apoptosis. The use of an inhibitor of late stages of autophagy restored TMZ-induced cell death (188). In accordance with this observation, chloroquine and hydroxychloroquine (HCQ) are being considered promising adjuvants in GBM therapy and are currently under phase III clinical trials (5, 189). Up until recently, these drugs were thought to improve median survival on GBM patients through autophagy inhibition. However, the maximum tolerated dose of HCQ was ineffective for autophagy inhibition, demonstrating the drug exerts its antitumoral effects through unknown mechanisms (190). Ultimately, the question of whether activation of autophagy would be beneficial or detrimental as a possible treatment for GBM remains unanswered. Knowledge on the crosstalk of autophagy with other cellular and molecular mechanisms can enlighten the discussion of how induction or suppression of this process should be safely applied in GBM treatment.

The comprehension of the metabolic features of GBM, highly associated with autophagic process, may contribute to circumvent the challenges faced in anti-tumor therapy. In fact, metabolic reprogramming is one of the hallmarks of cancer (191) and, in GBM, it fuels tumor survival, proliferation and invasion (192). Specially for this type of cancer, the upregulated WNT and AKT/mTOR signaling pathways are directly or indirectly involved with regulation of a diverse range of metabolic pathways (192, 193), intricately regulating autophagy processes and EMT.

As discussed in *Macroautophagy in Glioblastoma*, an overall downregulation of mTOR signaling exerted by MA was noted, leading to β-catenin degradation and decrease in WNT signaling (7, 144). On the other hand, mA is important for canonical WNT signaling activation. Evidence suggests methionine is required for the WNT-induced mA through the universal methyl donor S-adenosylmethionine (SAM), which would assist the internalization of GSK3 into late endosomes/MVBs (194). SAM is a known hub metabolite generated through one-carbon metabolism, which involves both folate and methionine cycles (195). Recently, WNT signaling has been emerging as a key regulator of cellular endocytic capacity and studies show endocytosis of extracellular proteins could provide cancer cells with recycle of nutrients required to grow, leading to adaptation and survival in a hostile microenvironment (64). Albrecht et al. (194) demonstrated methionine and SAM are necessary for this WNT-induced endolysosomal activity and extracellular protein degradation (194). In that sense, one-carbon metabolism has been shown to contribute to the *de novo* synthesis of purines and pyrimidines, in addition to nucleotide salvage in several tumors (193). In GBM, the scavenging of hypoxanthine is being considered as a reason for the resistance to anti-folate therapy (193). Furthermore, nearly half of GBM tumors have deleted 5-methylthioadenosine phosphorylase (MTAP), a key enzyme in the methionine salvage pathway (196). This enhances the dependence of the tumor cells for PRMT5 and studies have demonstrated PRMT5 inhibitors show antitumor effects against GBM (197–200). As such, PRMT5 inhibitors are currently being tested in clinical trials in GBM and several solid tumors (NCT02783300).

As one type of autophagic process, CMA has great impact on glucose metabolism (201). Studies with CMA-incompetent mice showed increased glycolysis in hepatocytes, due to decreased degradation of the glycolytic enzymes pyruvate kinase (PK), enolase 1 (eno1) and aldolase A (aldoA) (202). The glycolytic enzymes hexokinase 2 (HK2) and the M2 isoform of pyruvate kinase (PKM2) were also shown to be substrates of CMA degradation, in ovarian and non-small cell lung cancers, respectively (203, 204). GBM significantly boosts glycolysis for energy production through transcriptional and allosteric upregulation of key glycolytic enzymes such as HK2, enolase 2 (eno2) and phosphofructokinase (PFK), while decreasing the entering of pyruvate to the TCA cycle through inhibition of PDH (for more details on GBM metabolism, refer to 193). Interestingly, WNT3A/LRP5 signaling was shown to increase glycolysis during osteoblast differentiation through induction of the glycolytic enzymes GLUT1, HK2, PFK, PDK1 and others (184). Additionally, mTORC2 was shown to influence glycolytic metabolism in GBM through c-MYC activation (205). It would be interesting to evaluate whether canonical WNT signaling could be involved with glycolytic metabolism and CMA regulation in GBM cells, since the canonical WNT pathway is also upregulated in this tumor. Thus, in contrast to the possibility of CMA and MA hindering GBM metabolism and invasiveness, activation of mA may be a strategy employed by this tumor to survive in the surrounding microenvironment.

Given the importance of WNT signaling in GBM biology, the use of WNT pathway inhibitors are being evaluated for GBM therapy [reviewed in (206)]. LGK974 is a promising small-molecule inhibitor that interferes with the palmitoylation of WNTs, an indispensable step for their secretion and consequent binding to receptors. LGK97 was recently shown to have a synergistic effect with TMZ *in vitro*, reducing the clonogenic potential, with decreased expression of CD133, Nestin and SOX2. Importantly, these effects were shown to be independent of O^6-alkylguanine DNA alkyltransferase (*MGMT*) promoter methylation status (207). Some WNT signaling inhibitors are currently being tested in clinical trials, such as Enzastaurin and alisertib. Enzastaurin inhibits phosphorylation of GSK3 and AKT, through protein kinase Cβ inhibition. However, a phase II trial of Enzastaurin in combination with bevacizumab had response and progression free survival similar to bevacizumab monotherapy, although the drug was well-tolerated (208). Alisertib is an Aurora A kinase (AURKA) inhibitor that decreased WNT signaling in GBM *in vivo* and *in vitro*. AURKA interferes with GSK3/axin/β-catenin through interaction with axin. In patient-derived orthotopic models of GBM resistant to bevacizumab, alisertib prolonged survival, induced mitotic arrest and decreased histone H3 phosphorylation (209). In a recent phase I clinical trial for GBM patients, alisertib was safe and well tolerated (210). Hence, although WNT signaling inhibitors show promising therapeutic rationale and results against GBM, the intricate crosstalk between EMT-autophagy-WNT needs further evaluation to gain a clearer understanding of

how these processes and signaling pathways may be used against GBM.

CONCLUDING REMARKS

GBM is a highly heterogeneous and invasive solid tumor and, although there has been certain progress in the search for new therapeutic strategies, treatment of primary and recurrent GBM still remains a great challenge. Several of the signaling and metabolic abnormalities of GBMs are well described, especially those involving WNT, mTOR and EMT (**Figure 2**). However, the contributions of autophagic processes to GBM biology are largely unknown and debated, with clinical trials aiming at apparent opposing objectives. Some focus on autophagy inhibition, while others target mTOR inhibition to stimulate autophagy (211).

Moreover, as a highly heterogeneous tumor, GBM cells in different tumor layers may present distinct autophagic and EMT phenotypes that confer them with the molecular tools necessary to survive, proliferate, and evade therapy. As such, single-cell omics technologies are greatly contributing to the understanding of GBM heterogeneity and identification of subpopulations in the tumor bulk. However, it is crucial to first understand the molecular control and role of autophagy and EMT in GBM, to add to the identification of subpopulations of cells that would be the best target to overcome this disease.

As discussed in the present review, the WNT/β-catenin pathway plays a major role on EMT induction and invasiveness in GBM, while contributing to inhibition of MA (**Figure 2**). Interestingly, mA and one carbon metabolism are facilitators of β-catenin activation, and one carbon metabolism has been viewed as a possible therapeutic target in GBM (193). CMA was linked to acquired TMZ resistance in GBM cells, with very few reports about CMA in this tumor. Importantly, CMA assists the metabolic regulation of glycolytic enzymes and lipolysis and can also inhibit MA *via* p62 and HIF-1α degradation (**Figure 2**). Thus, there is an increasing demand for investigating mA, CMA and MA separately from each other in cancer and GBM (**Figure 3**). This could unveil the intricate relationship between these different autophagic processes, and

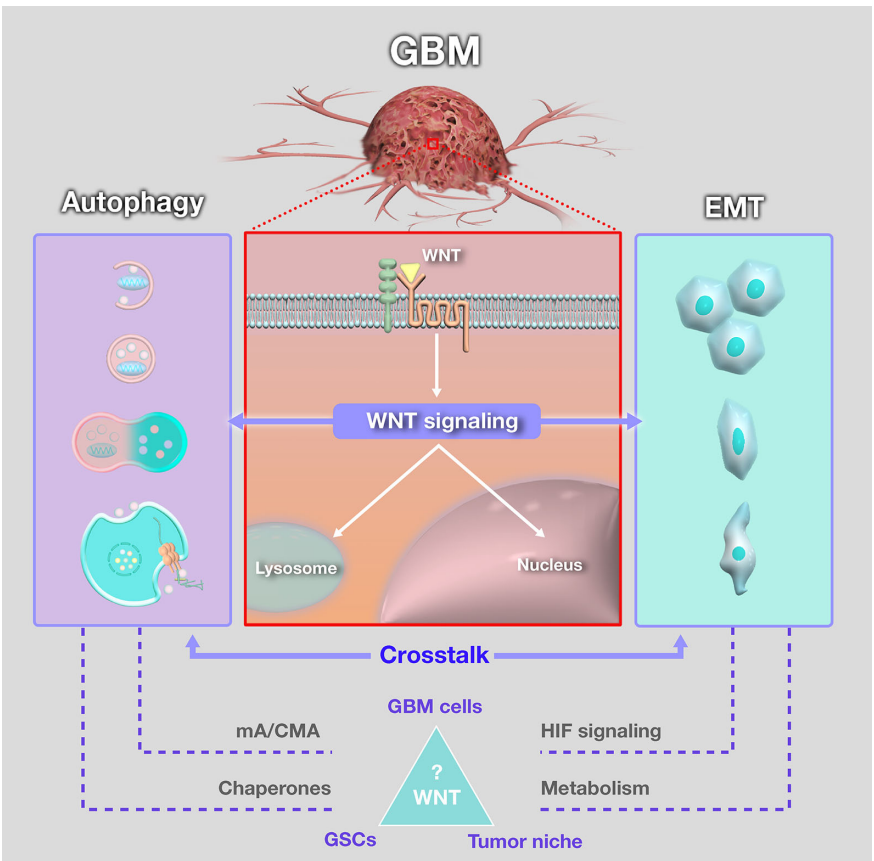


FIGURE 3 | Schematic summary of the crosstalk between WNT signaling, autophagy and epithelial-mesenchymal transition (EMT) in glioblastoma (GBM). Despite the extensively discussed relevance, the importante of WNT pathways in the crosstalk between autophagy and EMT in GBM is still surrounded by open questions. Among them, the role of microautophagy (mA) and chaperone-mediated autophagy (CMA) is largely unknown. Moreover, function of chaperones in this context might represent a target for crosstalk regulation in GBM, in glioblastoma stem-like cells (GSCs), tumor bulk and tumor niche, and warrant further investigation. Accordingly, the role of HIF signaling and metabolic reprogramming on this crosstalk should also be a subject for future research efforts.

how they influence cancer metabolism, aggressiveness, and invasion. Although these processes have overlapping signaling control, they can be activated or inhibited separately, which could confer tumor cells with a vast array of possible responses to survive and thrive even in harsh microenvironments.

AUTHOR CONTRIBUTIONS

Review conceptualization: BPC and MHL. Writing: BPC, CFLF, RPI, RNA, MIME, MBP, MCSS, JMB, GLRM, and GC. Review and editing: BPC, CFLF, RNA, MIME, MBP, MCSS, JMB, and MHL. Post-revision writing: BPC, CFLF, JMB, MCSS, GC, and GLRM. Post-revision editing: BPC, CFLF, and MHL. Figure conceptualization: BPC and MHL. Figure design: MIME. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Processes numbers: BPC: 2019/14952-0; CFLF: 2019/14741-9; RPI: 2019/12710-9; MBP: 2017/26158-0; MIME: 2019/11097-1; RNA: 2020/04687-4; MCSS: 2019/06971-4; JMB: 2020/07450-5; GLRM: 2019/14552-1; MHL: 2018/15557-4;) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Process number: JMB: 101796/2020-0).

REFERENCES

1. Liu EY, Ryan KM. Autophagy and cancer–issues we need to digest. *J Cell Sci* (2012) 125(Pt 10):2349–58. doi: 10.1242/jcs.093708
2. Kimmelman AC, White E. Autophagy and Tumor Metabolism. *Cell Metab* (2017) 25(5):1037–43. doi: 10.1016/j.cmet.2017.04.004
3. Nazio F, Bordi M, Cianfanelli V. Autophagy and cancer stem cells: molecular mechanisms and therapeutic applications. *Cell Death Differ* (2019) 26(4):690–702. doi: 10.1038/s41418-019-0292-y
4. Kaza N, Kohli L, Roth KA. Autophagy in brain tumors: a new target for therapeutic intervention. *Brain Pathol (Zurich Switzerland)* (2012) 22 (1):89–98. doi: 10.1111/j.1750-3639.2011.00544.x
5. Colella B, Faienza F, Di Bartolomeo S. EMT regulation by autophagy: a new perspective in glioblastoma biology. *Cancers* (2019) 11(3):312. doi: 10.3390/cancers11030312
6. Rojas-Sanchez G, Cotzomi-Ortega I, Pazos-Salazar NG, Reyes-Leyva J, Maycotte P. Autophagy and Its Relationship to Epithelial to Mesenchymal Transition: When Autophagy Inhibition for Cancer Therapy Turns Counterproductive. *Biology (Basel)* (2019) 8(4). doi: 10.3390/biology8040071
7. Chen H-T, Liu H, Mao M-J, Tan Y, Mo X-Q, Meng X-J, et al. Crosstalk between autophagy and epithelial-mesenchymal transition and its application in cancer therapy. *Mol Cancer* (2019) 18(1):1–19. doi: 10.1186/s12943-019-1030-2
8. Dongre A, Weinberg RA. New insights into the mechanisms of epithelial–mesenchymal transition and implications for cancer. *Nat Rev Mol Cell Biol* (2019) 20(2):69–84. doi: 10.1038/s41580-018-0080-4
9. Yang J, Antin P, Berx G, Blanpain C, Brabletz T, Bronner M, et al. Guidelines and definitions for research on epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol* (2020) 21(6):341–52. doi: 10.1038/s41580-020-0237-9
10. Catalano M, D'Alessandro G, Lepore F, Corazzari M, Caldarola S, Valacca C, et al. Autophagy induction impairs migration and invasion by reversing EMT in glioblastoma cells. *Mol Oncol* (2015) 9(8):1612–25. doi: 10.1016/j.molonc.2015.04.016
11. Wirsching HG, Galanis E, Weller M. Glioblastoma. *Handb Clin Neurol* (2016) 134:381–97. doi: 10.1016/b978-0-12-802997-8.00023-2
12. Chen Z, Hambardzumyan D. Immune microenvironment in glioblastoma subtypes. *Front Immunol* (2018) 9:1004. doi: 10.3389/fimmu.2018.01004
13. Noch EK, Ramakrishna R, Magge R. Challenges in the Treatment of Glioblastoma: Multisystem Mechanisms of Therapeutic Resistance. *World Neurosurg* (2018) 116:505–17. doi: 10.1016/j.wneu.2018.04.022
14. Weller M, Le Rhun E. How we treat glioblastoma. *Curr Oncol Rep* (2019) 4 (Suppl 2):e000520. doi: 10.1136/esmoopen-2019-000520
15. Campos B, Olsen LR, Urup T, Poulsen H. A comprehensive profile of recurrent glioblastoma. *Oncogene* (2016) 35(45):5819–25. doi: 10.1038/onc.2016.85
16. Batash R, Asna N, Schaffer P, Francis N, Schaffer M. Glioblastoma multiforme, diagnosis and treatment; recent literature review. *Curr Med Chem* (2017) 24(27):3002–9. doi: 10.2174/0929867324666170516123206
17. Sattiraju A, Sai KKS, Mintz A. Glioblastoma Stem Cells and Their Microenvironment. *Adv Exp Med Biol* (2017) 1041:119–40. doi: 10.1007/978-3-319-69194-7_7
18. Hatiboglu MA, Wei J, Wu AS, Heimberger AB. Immune therapeutic targeting of glioma cancer stem cells. *Targeted Oncol* (2010) 5(3):217–27. doi: 10.1007/s11523-010-0151-8
19. Liebelt BD, Shingu T, Zhou X, Ren J, Shin SA, Hu J. Glioma Stem Cells: Signaling, Microenvironment, and Therapy. *Stem Cells Int* (2016) 2016:7849890. doi: 10.1155/2016/7849890
20. Tompa M, Kalovits F. Contribution of the Wnt Pathway to Defining Biology of Glioblastoma. *Neuromolecular Med* (2018) 20(4):437–51. doi: 10.1007/s12017-018-8514-x
21. Liu X, Wang L, Zhao S, Ji X, Luo Y, Ling F. β-Catenin overexpression in malignant glioma and its role in proliferation and apoptosis in glioblastma cells. *Med Oncol (Northwood London England)* (2011) 28(2):608–14. doi: 10.1007/s12032-010-9476-5
22. Yu CY, Liang GB, Du P, Liu YH. Lgr4 promotes glioma cell proliferation through activation of Wnt signaling. *Asian Pacific J Cancer Prevent: APJCP* (2013) 14(8):4907–11. doi: 10.7314/apjcp.2013.14.8.4907
23. Kamino M, Kishida M, Kibe T, Ikoma K, Iijima M, Hirano H, et al. Wnt-5a signaling is correlated with infiltrative activity in human glioma by inducing cellular migration and MMP-2. *Cancer Sci* (2011) 102(3):540–8. doi: 10.1111/j.1349-7006.2010.01815.x
24. Vassallo I, Zinn P, Lai M, Rajakannu P, Hamou MF, Hegi ME. WIF1 re-expression in glioblastoma inhibits migration through attenuation of non-canonical WNT signaling by downregulating the lncRNA MALAT1. *Oncogene* (2016) 35(1):12–21. doi: 10.1038/onc.2015.61
25. Wang M, Li Q, Yu S, Zhang Z, Qiu P, Zhang Y, et al. Coronin 3 Promotes the Development of Oncogenic Properties in Glioma Through the Wnt/β-Catenin Signaling Pathway. *OncoTarg Ther* (2020) 13:6661–73. doi: 10.2147/ott.s257001
26. Rajakulendran N, Rowland KJ, Selvadurai HJ, Ahmadi M, Park NI, Naumenko S, et al. Wnt and Notch signaling govern self-renewal and differentiation in a subset of human glioblastoma stem cells. *Genes Dev* (2019) 33(9-10):498–510. doi: 10.1101/gad.321968.118
27. Huang M, Zhang D. Wnt-mediated endothelial transformation into mesenchymal stem cell-like cells induces chemoresistance in glioblastoma. *Sci Transl Med* (2020) 12(532):eaay7522. doi: 10.1126/scitranslmed.aay7522
28. Manoranjan B, Chokshi C, Venugopal C, Subapanditha M, Savage N, Tatari N, et al. A CD133-AKT-Wnt signaling axis drives glioblastoma brain tumor-initiating cells. *Oncogene* (2020) 39(7):1590–9. doi: 10.1038/s41388-019-1086-x
29. Mecca C, Giambanco I, Donato R, Arcuri C. Targeting mTOR in Glioblastoma: Rationale and Preclinical/Clinical Evidence. *Dis Markers* (2018) 2018:9230479. doi: 10.1155/2018/9230479
30. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell* (2012) 149(2):274–93. doi: 10.1016/j.cell.2012.03.017
31. Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, et al. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell* (2006) 22(2):159–68. doi: 10.1016/j.molcel.2006.03.029

32. Li X, Wu C, Chen N, Gu H, Yen A, Cao L, et al. PI3K/Akt/mTOR signaling pathway and targeted therapy for glioblastoma. *Oncotarget* (2016) 7 (22):33440–50. doi: 10.18632/oncotarget.7961
33. Shimobayashi M, Hall MN. Making new contacts: the mTOR network in metabolism and signalling crosstalk. *Nat Rev Mol Cell Biol* (2014) 15(3):155–62. doi: 10.1038/nrm3757
34. Nusse R, Clevers H. Wnt/β-Catenin Signaling, Disease, and Emerging Therapeutic Modalities. *Cell* (2017) 169(6):985–99. doi: 10.1016/j.cell.2017.05.016
35. Lee Y, Lee JK, Ahn SH, Lee J, Nam DH. WNT signaling in glioblastoma and therapeutic opportunities. *Lab Investigation; J Tech Methods Pathol* (2016) 96(2):137–50. doi: 10.1038/labinvest.2015.140
36. Cruciat C-M, Niehrs C. Secreted and transmembrane wnt inhibitors and activators. *Cold Spring Harbor Perspect Biol* (2013) 5(3):a015081. doi: 10.1101/cshperspect.a015081
37. Niehrs C. Function and biological roles of the Dickkopf family of Wnt modulators. *Oncogene* (2006) 25(57):7469–81. doi: 10.1038/sj.onc.1210054
38. Katase N, Nagano K, Fujita S. DKK3 expression and function in head and neck squamous cell carcinoma and other cancers. *J Oral Biosci* (2020) 62 (1):9–15. doi: 10.1016/j.job.2020.01.008
39. Li J, Gao Y, Yue W. The Clinical Diagnostic and Prognostic Value of Dickkopf-1 in Cancer. *Cancer Manag Res* (2020) 12:4253–60. doi: 10.2147/cmar.s254596
40. Esteve P, Bovolenta P. The advantages and disadvantages of sfrp1 and sfrp2 expression in pathological events. *Tohoku J Exp Med* (2010) 221(1):11–7. doi: 10.1620/tjem.221.11
41. Hu B, Wang Q, Wang YA, Hua S, Sauvé CG, Ong D, et al. Epigenetic Activation of WNT5A Drives Glioblastoma Stem Cell Differentiation and Invasive Growth. *Cell* (2016) 167(5):1281–95.e18. doi: 10.1016/j.cell.2016.10.039
42. Gao X, Mi Y, Ma Y, Jin W. LEF1 regulates glioblastoma cell proliferation, migration, invasion, and cancer stem-like cell self-renewal. *Tumour Biol: J Int Soc Oncodevelopmental Biol Med* (2014) 35(11):11505–11. doi: 10.1007/s13277-014-2466-z
43. Santiago L, Daniels G, Wang D, Deng FM, Lee P. Wnt signaling pathway protein LEF1 in cancer, as a biomarker for prognosis and a target for treatment. *Am J Cancer Res* (2017) 7(6):1389–406.
44. Adamo A, Fiore D, De Martino F, Roscigno G, Affinito A, Donnarumma E, et al. RYK promotes the stemness of glioblastoma cells via the WNT/β-catenin pathway. *Oncotarget* (2017) 8(8):13476. doi: 10.18632/oncotarget.14564
45. Zhang N, Wei P, Gong A, Chiu WT, Lee HT, Colman H, et al. FoxM1 promotes β-catenin nuclear localization and controls Wnt target-gene expression and glioma tumorigenesis. *Cancer Cell* (2011) 20(4):427–42. doi: 10.1016/j.ccr.2011.08.016
46. Lee Y, Kim KH, Kim DG, Cho HJ, Kim Y, Rheey J, et al. FoxM1 Promotes Stemness and Radio-Resistance of Glioblastoma by Regulating the Master Stem Cell Regulator Sox2. *PloS One* (2015) 10(10):e0137703. doi: 10.1371/journal.pone.0137703
47. Foltz G, Yoon JG, Lee H, Ma L, Tian Q, Hood L, et al. Epigenetic regulation of wnt pathway antagonists in human glioblastoma multiforme. *Genes Cancer* (2010) 1(1):81–90. doi: 10.1177/1947601909356103
48. Schiefer L, Visweswaran M, Perumal V, Arfuso F, Groth D, Newsholme P, et al. Epigenetic regulation of the secreted frizzled-related protein family in human glioblastoma multiforme. *Cancer Gene Ther* (2014) 21(7):297–303. doi: 10.1038/cgt.2014.30
49. Bhuvanalakshmi G, Gamit N, Patil M, Arfuso F, Sethi G, Dharmarajan A, et al. Stemness, pluripotentiality, and Wnt antagonism: sFRP4, a Wnt antagonist mediates pluripotency and stemness in glioblastoma. *Cancers* (2019) 11(1):25. doi: 10.3390/cancers11010025
50. Roos A, Ding Z, Loftus JC, Tran NL. Molecular and Microenvironmental Determinants of Glioma Stem-Like Cell Survival and Invasion. *Front Oncol* (2017) 7:120:120. doi: 10.3389/fonc.2017.00120
51. Fonseca AC, Romão L, Amaral RF, Assad Kahn S, Lobo D, Martins S, et al. Microglial stress inducible protein 1 promotes proliferation and migration in human glioblastoma cells. *Neuroscience* (2012) 200:130–41. doi: 10.1016/j.neuroscience.2011.10.025
52. Zhang I, Alizadeh D, Liang J, Zhang L, Gao H, Song Y, et al. Characterization of Arginase Expression in Glioma-Associated Microglia and Macrophages. *PloS One* (2016) 11(12):e0165118. doi: 10.1371/journal.pone.0165118
53. Matias D, Dubois LG, Pontes B, Rosário L, Ferrer VP, Balça-Silva J, et al. GBM-Derived Wnt3a Induces M2-Like Phenotype in Microglial Cells Through Wnt/β-Catenin Signaling. *Mol Neurobiol* (2019) 56(2):1517–30. doi: 10.1007/s12035-018-1150-5
54. Sharma P, Hu-Lieskovan S, Wargo JA, Ribas A. Primary, Adaptive, and Acquired Resistance to Cancer Immunotherapy. *Cell* (2017) 168(4):707–23. doi: 10.1016/j.cell.2017.01.017
55. Du L, Lee JH, Jiang H, Wang C, Wang S, Zheng Z, et al. β-Catenin induces transcriptional expression of PD-L1 to promote glioblastoma immune evasion. *J Exp Med* (2020) 217(11):e20191115. doi: 10.1084/jem.20191115
56. Zhang L, Liu H, Mu X, Cui J, Peng Z. Dysregulation of Fra1 expression by Wnt/β-catenin signalling promotes glioma aggressiveness through epithelial-mesenchymal transition. *Biosci Rep* (2017) 37(2):BSR20160643. doi: 10.1042/bsr20160643
57. Lefebvre C, Legouis R, Culetto E. ESCRT and autophagies: Endosomal functions and beyond. *Semin Cell Dev Biol* (2018) 74:21–8. doi: 10.1016/j.semcdb.2017.08.014
58. Parzych KR, Klionsky DJ. An overview of autophagy: morphology, mechanism, and regulation. *Antioxid Redox Signaling* (2014) 20(3):460–73. doi: 10.1089/ars.2013.5371
59. Drews U. Cholinesterase in embryonic development. *Prog Histochem Cytochem* (1975) 7(3):1–52. doi: 10.1016/S0079-6336(75)80008-8
60. Sakai M, Ogawa K. Energy-dependent lysosomal wrapping mechanism (LWM) during autophagolysosome formation. *Histochemistry* (1982) 76 (4):479–88. doi: 10.1007/bf00489903
61. Sahu R, Kaushik S, Clement CC, Cannizzo ES, Scharf B, Follenzi A, et al. Microautophagy of cytosolic proteins by late endosomes. *Dev Cell* (2011) 20 (1):131–9. doi: 10.1016/j.devcel.2010.12.003
62. Sato M, Seki T, Konno A, Hirai H, Kurauchi Y, Hisatsune A, et al. Rapamycin activates mammalian microautophagy. *J Pharmacol Sci* (2019) 140(2):201–4. doi: 10.1016/j.jphs.2019.05.007
63. Tejeda-Muñoz N, Albrecht LV, Bui MH, De Robertis EM. Wnt canonical pathway activates macropinocytosis and lysosomal degradation of extracellular proteins. *Proc Natl Acad Sci U States America* (2019) 116 (21):10402–11. doi: 10.1073/pnas.1903506116
64. Albrecht LV, Ploper D, Tejeda-Muñoz N, De Robertis EM. Arginine methylation is required for canonical Wnt signaling and endolysosomal trafficking. *Proc Natl Acad Sci* (2018) 115(23):E5317–E25. doi: 10.1073/pnas.1804091115
65. Cannizzo ES, Clement CC, Morozova K, Valdor R, Kaushik S, Almeida LN, et al. Age-related oxidative stress compromises endosomal proteostasis. *Cell Rep* (2012) 2(1):136–49. doi: 10.1016/j.celrep.2012.06.005
66. Uytterhoeven V, Lauwers E, Maes I, Miskiewicz K, Melo MN, Swerts J, et al. Hsc70-4 Deforms Membranes to Promote Synaptic Protein Turnover by Endosomal Microautophagy. *Neuron* (2015) 88(4):735–48. doi: 10.1016/j.neuron.2015.10.012
67. Huber LA, Teis D. Lysosomal signaling in control of degradation pathways. *Curr Opin Cell Biol* (2016) 39:8–14. doi: 10.1016/j.ceb.2016.01.006
68. Jarrold J, Davies CC. PRMTs and Arginine Methylation: Cancer's Best-Kept Secret? *Trends Mol Med* (2019) 25(11):993–1009. doi: 10.1016/j.molmed.2019.05.007
69. Dong F, Li Q, Yang C, Huo D, Wang X, Ai C, et al. PRMT2 links histone H3R8 asymmetric dimethylation to oncogenic activation and tumorigenesis of glioblastoma. *Nat Commun* (2018) 9(1):1–14. doi: 10.1038/s41467-018-06968-7
70. Banasavadi-Siddegowda Y, Russell L, Frair E, Karkhanis V, Relation T, Yoo J, et al. PRMT5–PTEN molecular pathway regulates senescence and self-renewal of primary glioblastoma neurosphere cells. *Oncogene* (2017) 36 (2):263–74. doi: 10.1038/onc.2016.199
71. Holmes B, Benavides-Serrato A, Saunders JT, Landon KA, Schreck AJ, Nishimura RN, et al. The protein arginine methyltransferase PRMT5 confers therapeutic resistance to mTOR inhibition in glioblastoma. *J Neurooncol* (2019) 145(1):11–22. doi: 10.1007/s11060-019-03274-0
72. Zhang C, Cuervo AM. Restoration of chaperone-mediated autophagy in aging liver improves cellular maintenance and hepatic function. *Nat Med* (2008) 14(9):959–65. doi: 10.1038/nm.1851
73. Arias E, Cuervo AM. Chaperone-mediated autophagy in protein quality control. *Curr Opin Cell Biol* (2011) 23(2):184–9. doi: 10.1016/j.ceb.2010.10.009

74. Chiang H-L, Plant C, Dice J. A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. *Science* (4928) 1989) 246:382–5. doi: 10.1126/science.2799391
75. Kaushik S, Cuervo AM. The coming of age of chaperone-mediated autophagy. *Nat Rev Mol Cell Biol* (2018) 19(6):365–81. doi: 10.1038/s41580-018-0001-6
76. Massey AC, Kaushik S, Sovak G, Kiffin R, Cuervo AM. Consequences of the selective blockage of chaperone-mediated autophagy. *Proc Natl Acad Sci U States America* (2006) 103(15):5805–10. doi: 10.1073/pnas.0507436103
77. Agarraberes FA, Dice JF. A molecular chaperone complex at the lysosomal membrane is required for protein translocation. *J Cell Sci* (2001) 114 (13):2491–9.
78. Bandyopadhyay U, Kaushik S, Varticovski L, Cuervo AM. The chaperone-mediated autophagy receptor organizes in dynamic protein complexes at the lysosomal membrane. *Mol Cell Biol* (2008) 28(18):5747–63. doi: 10.1128/MCB.02070-07
79. Cuervo A, Dice J. Unique properties of lamp2a compared to other lamp2 isoforms. *J Cell Sci* (2000) 113(24):4441–50.
80. Cuervo AM, Dice J. Regulation of lamp2a levels in the lysosomal membrane. *Traffic* (2000) 1(7):570–83. doi: 10.1034/j.1600-0854.2000.010707.x
81. Bandyopadhyay U, Sridhar S, Kaushik S, Kiffin R, Cuervo AM. Identification of regulators of chaperone-mediated autophagy. *Mol Cell* (2010) 39(4):535–47. doi: 10.1016/j.molcel.2010.08.004
82. Anguiano J, Garner TP, Mahalingam M, Das BC, Gavathiotis E, Cuervo AM. Chemical modulation of chaperone-mediated autophagy by retinoic acid derivatives. *Nat Chem Biol* (2013) 9(6):374. doi: 10.1038/nchembio.1230
83. Gomes LR, Menck CFM, Cuervo AM. Chaperone-mediated autophagy prevents cellular transformation by regulating MYC proteasomal degradation. *Autophagy* (2017) 13(5):928–40. doi: 10.1080/15548627.2017.1293767
84. Kon M, Kiffin R, Koga H, Chapochnick J, Macian F, Varticovski L, et al. Chaperone-mediated autophagy is required for tumor growth. *Sci Trans Med* (2011) 3(109):109ra17. doi: 10.1126/scitranslmed.3003182
85. Peng JQ, Han SM, Chen ZH, Yang J, Pei YQ, Bao C, et al. Chaperone-mediated autophagy regulates apoptosis and the proliferation of colon carcinoma cells. *Biochem Biophys Res Commun* (2020) 522(2):348–54. doi: 10.1016/j.bbrc.2019.11.081
86. Maiti P, Scott J, Sengupta D, Al-Gharaibeh A, Dunbar GL. Curcumin and Solid Lipid Curcumin Particles Induce Autophagy, but Inhibit Mitophagy and the PI3K-Akt/mTOR Pathway in Cultured Glioblastoma Cells. *Int J Mol Sci* (2019) 20(2):399. doi: 10.3390/ijms20020399
87. Lo Dico A, Martelli C, Diceglie C, Lucignani G, Ottobrini L. Hypoxia-Inducible Factor-1α Activity as a Switch for Glioblastoma Responsiveness to Temozolomide. *Front Oncol* (2018) 8:249:249. doi: 10.3389/fonc.2018.00249
88. Lo Dico A, Salvatore D. Intracellular Redox-Balance Involvement in Temozolomide Resistance-Related Molecular Mechanisms in Glioblastoma. *Cells* (2019) 8(11):1315. doi: 10.3390/cells8111315
89. Valdor R, Mocholi E, Botbol Y, Guerrero-Ros I, Chandra D, Koga H, et al. Chaperone-mediated autophagy regulates T cell responses through targeted degradation of negative regulators of T cell activation. *Nat Immunol* (2014) 15(11):1046–54. doi: 10.1038/ni.3003
90. Komiya Y, Habas R. Wnt signal transduction pathways. *Organogenesis* (2008) 4(2):68–75. doi: 10.4161/org.4.2.5851
91. Valdor R, García-Bernal D, Riquelme D, Martinez CM. Glioblastoma ablates pericytes antitumor immune function through aberrant up-regulation of chaperone-mediated autophagy. *Proc Natl Acad Sci U S A* (2019) 116 (41):20655–65. doi: 10.1073/pnas.1903542116
92. Iglesia RP, Fernandes CFL. Heat Shock Proteins in Glioblastoma Biology: Where Do We Stand? *Int J Mol Sci* (2019) 20(22):5794. doi: 10.3390/ijms20225794
93. Lopes MH, Santos TG, Rodrigues BR, Queiroz-Hazarbassanov N, Cunha IW, Wasilewska-Sampaio AP, et al. Disruption of prion protein-HOP engagement impairs glioblastoma growth and cognitive decline and improves overall survival. *Oncogene* (2015) 34(25):3305–14. doi: 10.1038/onc.2014.261
94. Iglesia RP, Prado MB, Cruz L, Martins VR, Santos TG, Lopes MH. Engagement of cellular prion protein with the co-chaperone Hsp70/90 organizing protein regulates the proliferation of glioblastoma stem-like cells. *Stem Cell Res Ther* (2017) 8(1):76. doi: 10.1186/s13287-017-0518-1
95. Wang H, Tian C, Sun J, Chen LN, Lv Y, Yang XD, et al. Overexpression of PLK3 Mediates the Degradation of Abnormal Prion Proteins Dependent on Chaperone-Mediated Autophagy. *Mol Neurobiol* (2017) 54(6):4401–13. doi: 10.1007/s12035-016-9985-0
96. Roth W, Grimmel C, Rieger L, Strik H, Takayama S, Krajewski S, et al. Bag-1 and Bcl-2 gene transfer in malignant glioma: modulation of cell cycle regulation and apoptosis. *Brain Pathol (Zurich Switzerland)* (2000) 10 (2):223–34. doi: 10.1111/j.1750-3639.2000.tb00256.x
97. Chen Y, Klionsky DJ. The regulation of autophagy–unanswered questions. *J Cell Sci* (2011) 124(2):161–70. doi: 10.1242/jcs.064576
98. Onorati AV, Dyczynski M, Ojha R, Amaravadi RK. Targeting autophagy in cancer. *Cancer* (2018) 124(16):3307–18. doi: 10.1002/cncr.31335
99. Hosokawa N, Sasaki T, Iemura S, Natsume T, Hara T, Mizushima N. Atg101, a novel mammalian autophagy protein interacting with Atg13. *Autophagy* (2009) 5(7):973–9. doi: 10.4161/auto.5.7.9296
100. Jung CH, Jun CB, Ro SH, Kim YM, Otto NM, Cao J, et al. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol Biol Cell* (2009) 20(7):1992–2003. doi: 10.1091/mbc.e08-12-1249
101. Lee JW, Park S, Takahashi Y, Wang HG. The association of AMPK with ULK1 regulates autophagy. *PloS One* (2010) 5(11):e15394. doi: 10.1371/journal.pone.0015394
102. Zhao M, Klionsky DJ. AMPK-dependent phosphorylation of ULK1 induces autophagy. *Cell Metab* (2011) 13(2):119–20. doi: 10.1016/j.cmet.2011.01.009
103. He C, Song H, Yorimitsu T, Monastyrska I, Yen WL, Legakis JE, et al. Recruitment of Atg9 to the preautophagosomal structure by Atg11 is essential for selective autophagy in budding yeast. *J Cell Biol* (2006) 175 (6):925–35. doi: 10.1083/jcb.200606084
104. Zhou C, Ma K, Gao R, Mu C, Chen L, Liu Q, et al. Regulation of mATG9 trafficking by Src- and ULK1-mediated phosphorylation in basal and starvation-induced autophagy. *Cell Res* (2017) 27(2):184–201. doi: 10.1038/cr.2016.146
105. Tanida I, Ueno T, Kominami E. LC3 and Autophagy. *Methods Mol Biol (Clifton NJ)* (2008) 445:77–88. doi: 10.1007/978-1-59745-157-4_4
106. Shvets E, Fass E, Scherz-Shouval R, Elazar Z. The N-terminus and Phe52 residue of LC3 recruit p62/SQSTM1 into autophagosomes. *J Cell Sci* (2008) 121(Pt 16):2685–95. doi: 10.1242/jcs.026005
107. Yang Z, Klionsky DJ. An overview of the molecular mechanism of autophagy. *Curr Topics Microbiol Immunol* (2009) 335:1–32. doi: 10.1007/978-3-642-00302-8_1
108. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. *J Pathol* (2010) 221(1):3–12. doi: 10.1002/path.2697
109. Tanaka Y, Guhde G, Suter A, Eskelinen EL, Hartmann D, Lüllmann-Rauch R, et al. Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. *Nature* (2000) 406(6798):902–6. doi: 10.1038/35022595
110. Jäger S, Bucci C, Tanida I, Ueno T, Kominami E, Saftig P, et al. Role for Rab7 in maturation of late autophagic vacuoles. *J Cell Sci* (2004) 117(Pt 20):4837–48. doi: 10.1242/jcs.01370
111. Yang K, Niu L, Bai Y, Le W. Glioblastoma: Targeting the autophagy in tumorigenesis. *Brain Res Bull* (2019) 153:334–40. doi: 10.1016/j.brainresbull.2019.09.012
112. Wang T, Chen Z, Zhang W. Regulation of autophagy inhibition and inflammatory response in glioma by Wnt signaling pathway. *Oncol Lett* (2017) 14(6):7197–200. doi: 10.3892/ol.2017.7103
113. Nàger M, Sallán MC, Visa A, Pushparaj C, Santacana M, Macià A, et al. Inhibition of WNT-CTNNB1 signaling upregulates SQSTM1 and sensitizes glioblastoma cells to autophagy blockers. *Autophagy* (2018) 14(4):619–36. doi: 10.1080/15548627.2017.1423439
114. Colella B, Faienza F, Carinci M, D'Alessandro G, Catalano M, Santoro A, et al. Autophagy induction impairs Wnt/β-catenin signalling through β-catenin relocalisation in glioblastoma cells. *Cell Signal* (2019) 53:357–64. doi: 10.1016/j.cellsig.2018.10.017
115. Gao C, Cao W, Bao L, Zuo W, Xie G, Cai T, et al. Autophagy negatively regulates Wnt signalling by promoting Dishevelled degradation. *Nat Cell Biol* (2010) 12(8):781–90. doi: 10.1038/ncb2082
116. Ziello JE, Jovin IS, Huang Y. Hypoxia-Inducible Factor (HIF)-1 regulatory pathway and its potential for therapeutic intervention in malignancy and ischemia. *Yale J Biol Med* (2007) 80(2):51–60.

117. Yang L, Lin C, Wang L, Guo H, Wang X. Hypoxia and hypoxia-inducible factors in glioblastoma multiforme progression and therapeutic implications. *Exp Cell Res* (2012) 318(19):2417–26. doi: 10.1016/j.yexcr.2012.07.017
118. Semenza GL. HIF-1 mediates metabolic responses to intratumoral hypoxia and oncogenic mutations. *J Clin Invest* (2013) 123(9):3664–71. doi: 10.1172/jci67230
119. DePavia A, Jonasch E, Liu X-D. Autophagy degrades hypoxia inducible factors. *Mol Cell Oncol* (2016) 3(2):e1104428. doi: 10.1080/23723556.2015.1104428
120. Liu XD, Yao J, Tripathi DN, Ding Z, Xu Y, Sun M, et al. Autophagy mediates HIF2α degradation and suppresses renal tumorigenesis. *Oncogene* (2015) 34 (19):2450–60. doi: 10.1038/onc.2014.199
121. Hu YL, DeLay M, Jahangiri A, Molinaro AM, Rose SD, Carbonell WS, et al. Hypoxia-induced autophagy promotes tumor cell survival and adaptation to antiangiogenic treatment in glioblastoma. *Cancer Res* (2012) 72(7):1773–83. doi: 10.1158/0008-5472.can-11-3831
122. Boso D, Rampazzo E, Zanon C, Bresolin S, Maule F, Porcù E, et al. HIF-1α/Wnt signaling-dependent control of gene transcription regulates neuronal differentiation of glioblastoma stem cells. *Theranostics* (2019) 9(17):4860. doi: 10.7150/thno.35882
123. Kim DH, Xing T, Yang Z, Dudek R, Lu Q, Chen YH. Epithelial Mesenchymal Transition in Embryonic Development, Tissue Repair and Cancer: A Comprehensive Overview. *J Clin Med* (2017) 7(1):1. doi: 10.3390/jcm7010001
124. Horejs CM. Basement membrane fragments in the context of the epithelial-to-mesenchymal transition. *Eur J Cell Biol* (2016) 95(11):427–40. doi: 10.1016/j.ejcb.2016.06.002
125. Iser IC, Pereira MB, Lenz G, Wink MR. The Epithelial-to-Mesenchymal Transition-Like Process in Glioblastoma: An Updated Systematic Review and In Silico Investigation. *Med Res Rev* (2017) 37(2):271–313. doi: 10.1002/med.21408
126. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol* (2014) 15(3):178–96. doi: 10.1038/nrm3758
127. Wheelock MJ, Shintani Y, Maeda M, Fukumoto Y, Johnson KR. Cadherin switching. *J Cell Sci* (2008) 121(Pt 6):727–35. doi: 10.1242/jcs.000455
128. Loh CY, Chai JY, Tang TF, Wong WF. The E-Cadherin and N-Cadherin Switch in Epithelial-to-Mesenchymal Transition: Signaling, Therapeutic Implications, and Challenges. *Cells* (2019) 8(10):1118. doi: 10.3390/cells8101118
129. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* (2009) 119(6):1420–8. doi: 10.1172/jci39104
130. Haensel D, Dai X. Epithelial-to-mesenchymal transition in cutaneous wound healing: Where we are and where we are heading. *Dev Dyn* (2018) 247 (3):473–80. doi: 10.1002/dvdy.24561
131. Pearson GW. Control of Invasion by Epithelial-to-Mesenchymal Transition Programs during Metastasis. *J Clin Med* (2019) 8(5):646. doi: 10.3390/jcm8050646
132. Ferrer-Vaquer A, Viotti M, Hadjantonakis AK. Transitions between epithelial and mesenchymal states and the morphogenesis of the early mouse embryo. *Cell Adhesion Migration* (2010) 4(3):447–57. doi: 10.4161/cam.4.3.10771
133. Kalluri R. EMT: when epithelial cells decide to become mesenchymal-like cells. *J Clin Invest* (2009) 119(6):1417–9. doi: 10.1172/jci39675
134. Mayor R, Theveneau E. The neural crest. *Dev (Cambridge England)* (2013) 140(11):2247–51. doi: 10.1242/dev.091751
135. Campbell K. Contribution of epithelial-mesenchymal transitions to organogenesis and cancer metastasis. *Curr Opin Cell Biol* (2018) 55:30–5. doi: 10.1016/j.ceb.2018.06.008
136. Lewis-Tuffin LJ, Rodriguez F, Giannini C, Scheithauer B, Necela BM, Sarkaria JN, et al. Misregulated E-cadherin expression associated with an aggressive brain tumor phenotype. *PloS One* (2010) 5(10):e13665. doi: 10.1371/journal.pone.0013665
137. Kubelt C, Hattermann K, Sebens S, Mehdorn HM, Held-Feindt J. Epithelial-to-mesenchymal transition in paired human primary and recurrent glioblastomas. *Int J Oncol* (2015) 46(6):2515–25. doi: 10.3892/ijo.2015.2944
138. Perego C, Vanoni C, Massari S, Raimondi A, Pola S, Cattaneo MG, et al. Invasive behaviour of glioblastoma cell lines is associated with altered organisation of the cadherin-catenin adhesion system. *J Cell Sci* (2002) 115 (Pt 16):3331–40.
139. Mahabir R, Tanino M, Elmansuri A, Wang L, Kimura T, Itoh T, et al. Sustained elevation of Snail promotes glial-mesenchymal transition after irradiation in malignant glioma. *Neuro Oncol* (2014) 16(5):671–85. doi: 10.1093/neuonc/not239
140. Itoh Y, Moriyama Y, Hasegawa T, Endo TA, Toyoda T, Gotoh Y. Scratch regulates neuronal migration onset via an epithelial-mesenchymal transition-like mechanism. *Nat Neurosci* (2013) 16(4):416–25. doi: 10.1038/nn.3336
141. Zhang J, Cai H, Sun L, Zhan P, Chen M, Zhang F, et al. LGR5, a novel functional glioma stem cell marker, promotes EMT by activating the Wnt/β-catenin pathway and predicts poor survival of glioma patients. *J Exp Clin Cancer Res: CR* (2018) 37(1):225. doi: 10.1186/s13046-018-0864-6
142. Liu Q, Guan Y, Li Z, Wang Y, Liu Y, Cui R, et al. miR-504 suppresses mesenchymal phenotype of glioblastoma by directly targeting the FZD7-mediated Wnt-β-catenin pathway. *J Exp Clin Cancer Res: CR* (2019) 38 (1):358. doi: 10.1186/s13046-019-1370-1
143. Zuccarini M, Giuliani P, Ziberi S, Carluccio M, Iorio PD, Caciagli F, et al. The Role of Wnt Signal in Glioblastoma Development and Progression: A Possible New Pharmacological Target for the Therapy of This Tumor. *Genes* (2018) 9(2):105. doi: 10.3390/genes9020105
144. Guo S, Liang X, Guo M, Zhang X, Li Z. Migration inhibition of water stress proteins from Nostoc commune Vauch. via activation of autophagy in DLD-1 cells. *Int J Biol Macromol* (2018) 119:669–76. doi: 10.1016/j.ijbiomac.2018.07.188
145. Colwell N, Larion M, Giles AJ, Seldomridge AN, Sizdahkhani S, Gilbert MR, et al. Hypoxia in the glioblastoma microenvironment: shaping the phenotype of cancer stem-like cells. *Neuro-oncology* (2017) 19(7):887–96. doi: 10.1093/neuonc/now258
146. Zhu H, Wang D, Zhang L, Xie X, Wu Y, Liu Y, et al. Upregulation of autophagy by hypoxia-inducible factor-1α promotes EMT and metastatic ability of CD133+ pancreatic cancer stem-like cells during intermittent hypoxia. *Oncol Rep* (2014) 32(3):935–42. doi: 10.3892/or.2014.3298
147. Qin W, Li C, Zheng W, Guo Q, Zhang Y, Kang M, et al. Inhibition of autophagy promotes metastasis and glycolysis by inducing ROS in gastric cancer cells. *Oncotarget* (2015) 6(37):39839–54. doi: 10.18632/oncotarget.5674
148. Huang W, Ding X, Ye H, Wang J, Shao J, Huang T. Hypoxia enhances the migration and invasion of human glioblastoma U87 cells through PI3K/Akt/mTOR/HIF-1α pathway. *Neuroreport* (2018) 29(18):1578–85. doi: 10.1097/wnr.0000000000001156
149. Deng M, Zhang W, Yuan L, Tan J, Chen Z. HIF-1a regulates hypoxia-induced autophagy via translocation of ANKRD37 in colon cancer. *Exp Cell Res* (2020) 395(1):112175. doi: 10.1016/j.yexcr.2020.112175
150. Tam SY, Wu VWC, Law HKW. Hypoxia-Induced Epithelial-Mesenchymal Transition in Cancers: HIF-1α and Beyond. *Front Oncol* (2020) 10:486:486. doi: 10.3389/fonc.2020.00486
151. Ghosh S, Paul A, Sen E. Tumor necrosis factor α-induced hypoxia-inducible factor 1α-β-catenin axis regulates major histocompatibility complex class I gene activation through chromatin remodeling. *Mol Cell Biol* (2013) 33 (14):2718–31. doi: 10.1128/mcb.01254-12
152. Mazumdar J, O'Brien WT, Johnson RS, LaManna JC, Chavez JC, Klein PS, et al. O2 regulates stem cells through Wnt/β-catenin signalling. *Nat Cell Biol* (2010) 12(10):1007–13. doi: 10.1038/ncb2102
153. Fu Y, Zheng S, Zheng Y, Huang R, An N, Liang A, et al. Glioma derived isocitrate dehydrogenase-2 mutations induced up-regulation of HIF-1α and β-catenin signaling: possible impact on glioma cell metastasis and chemo-resistance. *Int J Biochem Cell Biol* (2012) 44(5):770–5. doi: 10.1016/j.biocel.2012.01.017
154. Náger M, Santacana M, Bhardwaj D, Valls J, Ferrer I, Nogués P, et al. Nuclear phosphorylated Y142 β-catenin accumulates in astrocytomas and glioblastomas and regulates cell invasion. *Cell Cycle (Georgetown Tex)* (2015) 14(22):3644–55. doi: 10.1080/15384101.2015.1104443
155. Rampazzo E, Persano L, Pistollato F, Moro E, Frasson C, Porazzi P, et al. Wnt activation promotes neuronal differentiation of glioblastoma. *Cell Death Dis* (2013) 4(2):e500. doi: 10.1038/cddis.2013.32
156. Shin S, Im HJ, Kwon YJ, Ye DJ, Baek HS, Kim D, et al. Human steroid sulfatase induces Wnt/β-catenin signaling and epithelial-mesenchymal transition by upregulating Twist1 and HIF-1α in human prostate and

cervical cancer cells. *Oncotarget* (2017) 8(37):61604–17. doi: 10.18632/oncotarget.18645
157. Ha JH, Ward JD, Radhakrishnan R, Jayaraman M, Song YS, Dhanasekaran DN. Lysophosphatidic acid stimulates epithelial to mesenchymal transition marker Slug/Snail2 in ovarian cancer cells via Gαi2, Src, and HIF1α signaling nexus. *Oncotarget* (2016) 7(25):37664–79. doi: 10.18632/oncotarget.9224
158. Goossens S, Vandamme N, Van Vlierberghe P, Berx G. EMT transcription factors in cancer development re-evaluated: Beyond EMT and MET. *Biochim Biophys Acta Rev Cancer* (2017) 1868(2):584–91. doi: 10.1016/j.bbcan.2017.06.006
159. Liu T, Zou XZ, Huang N, Ge XY, Yao MZ, Liu H, et al. Down-regulation of miR-204 attenuates endothelial-mesenchymal transition by enhancing autophagy in hypoxia-induced pulmonary hypertension. *Eur J Pharmacol* (2019) 863:172673. doi: 10.1016/j.ejphar.2019.172673
160. Bagherian A, Mardani R, Roudi B, Taghizadeh M, Banfshe HR, Ghaderi A, et al. Combination therapy with nanomicellar-curcumin and temozolomide for in vitro therapy of glioblastoma multiforme via Wnt signaling pathways. *J Mol Neurosci* (2020) 70(10):1471–83. doi: 10.1007/s12031-020-01639-z
161. Shen X, Wu S, Zhang J, Li M, Xu F, Wang A, et al. Wild−type IDH1 affects cell migration by modulating the PI3K/AKT/mTOR pathway in primary glioblastoma cells. *Mol Med Rep* (2020) 22(3):1949–57. doi: 10.3892/mmr.2020.11250
162. Liu M, Dai B, Kang SH, Ban K, Huang FJ, Lang FF, et al. FoxM1B is overexpressed in human glioblastomas and critically regulates the tumorigenicity of glioma cells. *Cancer Res* (2006) 66(7):3593–602. doi: 10.1158/0008-5472.can-05-2912
163. Dai B, Kang S-H, Gong W, Liu M, Aldape K, Sawaya R, et al. Aberrant FoxM1B expression increases matrix metalloproteinase-2 transcription and enhances the invasion of glioma cells. *Oncogene* (2007) 26(42):6212–9. doi: 10.1038/sj.onc.1210443
164. Zhang C, Han X, Xu X, Zhou Z, Chen X, Tang Y, et al. FoxM1 drives ADAM17/EGFR activation loop to promote mesenchymal transition in glioblastoma. *Cell Death Dis* (2018) 9(5):469. doi: 10.1038/s41419-018-0482-4
165. Chen Y, Li Y, Xue J, Gong A, Yu G, Zhou A, et al. Wnt-induced deubiquitination FoxM1 ensures nucleus β-catenin transactivation. *EMBO J* (2016) 35(6):668–84. doi: 10.15252/embj.201592810
166. Guo L, Ding Z, Huang N, Huang Z, Zhang N, Xia Z. Forkhead Box M1 positively regulates UBE2C and protects glioma cells from autophagic death. *Cell Cycle (Georgetown Tex)* (2017) 16(18):1705–18. doi: 10.1080/15384101.2017.1356507
167. Pulvirenti T, Van Der Heijden M, Droms LA, Huse JT, Tabar V, Hall A. Dishevelled 2 signaling promotes self-renewal and tumorigenicity in human gliomas. *Cancer Res* (2011) 71(23):7280–90. doi: 10.1158/0008-5472.can-11-1531
168. Kafka A, Tomas D. Expression Levels and Localizations of DVL3 and sFRP3 in Glioblastoma. *Dis Markers* (2017) 2017:9253495. doi: 10.1155/2017/9253495
169. Shevchenko V, Arnotskaya N, Zaitsev S, Sharma A, Sharma HS, Bryukhovetskiy A, et al. Proteins of Wnt signaling pathway in cancer stem cells of human glioblastoma. *Int Rev Neurobiol* (2020) 151:185–200. doi: 10.1016/bs.irn.2020.03.006
170. Kim DH, Kim EJ, Kim DH, Park SW. Dact2 is involved in the regulation of epithelial-mesenchymal transition. *Biochem Biophys Res Commun* (2020) 524(1):190–7. doi: 10.1016/j.bbrc.2019.12.090
171. Tan Y, Li QM, Huang N, Cheng S, Zhao GJ, Chen H, et al. Upregulation of DACT2 suppresses proliferation and enhances apoptosis of glioma cell via inactivation of YAP signaling pathway. *Cell Death Dis* (2017) 8(8):e2981. doi: 10.1038/cddis.2017.385
172. Ma B, Cao W, Li W, Gao C, Qi Z, Zhao Y, et al. Dapper1 promotes autophagy by enhancing the Beclin1-Vps34-Atg14L complex formation. *Cell Res* (2014) 24(8):912–24. doi: 10.1038/cr.2014.84
173. Ren Y, Chen Y, Liang X, Lu Y, Pan W, Yang M. MiRNA-638 promotes autophagy and malignant phenotypes of cancer cells via directly suppressing DACT3. *Cancer Lett* (2017) 390:126–36. doi: 10.1016/j.canlet.2017.01.009
174. Arbab AS, Ali MM. Glioblastoma: Targeting Angiogenesis and Tyrosine Kinase Pathways. *Novel Approaches Cancer Study* (2020) 4(5):398.
175. Winkler F, Osswald M, Wick W. Anti-Angiogenics: Their Role in the Treatment of Glioblastoma. *Oncol Res Treat* (2018) 41(4):181–6. doi: 10.1159/000488258
176. Miranda-Gonçalves V, Cardoso-Carneiro D, Valbom I, Cury FP, Silva VA, Granja S, et al. Metabolic alterations underlying Bevacizumab therapy in glioblastoma cells. *Oncotarget* (2017) 8(61):103657–70. doi: 10.18632/oncotarget.21761
177. Wickström SA, Alitalo K, Keski-Oja J. Endostatin associates with integrin alpha5beta1 and caveolin-1, and activates Src via a tyrosyl phosphatase-dependent pathway in human endothelial cells. *Cancer Res* (2002) 62(19):5580–9.
178. Sudhakar A, Sugimoto H, Yang C, Lively J, Zeisberg M, Kalluri R. Human tumstatin and human endostatin exhibit distinct antiangiogenic activities mediated by alpha v beta 3 and alpha 5 beta 1 integrins. *Proc Natl Acad Sci U States America* (2003) 100(8):4766–71. doi: 10.1073/pnas.0730882100
179. Hanai J, Gloy J, Karumanchi SA, Kale S, Tang J, Hu G, et al. Endostatin is a potential inhibitor of Wnt signaling. *J Cell Biol* (2002) 158(3):529–39. doi: 10.1083/jcb.200203064
180. Hu P, Ma L, Wu ZQ, Zheng GY, Li JT. Effect of endostatin on proliferation, invasion and epithelial-mesenchymal transition of basal cell carcinoma cell A431. *Eur Rev Med Pharmacol Sci* (2019) 23(2):877–84. doi: 10.26355/eurrev_201901_16902
181. Nguyen TM, Subramanian IV, Xiao X, Ghosh G, Nguyen P, Kelekar A, et al. Endostatin induces autophagy in endothelial cells by modulating Beclin 1 and beta-catenin levels. *J Cell Mol Med* (2009) 13(9b):3687–98. doi: 10.1111/j.1582-4934.2009.00722.x
182. Chu C-W, Ko H-J, Chou C-H, Cheng T-S, Cheng H-W, Liang Y-H, et al. Thioridazine enhances P62-mediated autophagy and apoptosis through Wnt/β-catenin signaling pathway in glioma cells. *Int J Mol Sci* (2019) 20 (3):473. doi: 10.3390/ijms20030473
183. Chen H, Lu Q, Chen C, Di Y, Yn L, Min W, et al. β−catenin regulates effects of miR−24 on the viability and autophagy of glioma cells. *Exp Ther Med* (2019) 18(2):1285–90. doi: 10.3892/etm.2019.7680
184. Esen E, Chen J, Karner CM, Okunade AL, Patterson BW, Long F. WNT-LRP5 signaling induces Warburg effect through mTORC2 activation during osteoblast differentiation. *Cell Metab* (2013) 17(5):745–55. doi: 10.1016/j.cmet.2013.03.017
185. Kanzawa T, Zhang L, Xiao L, Germano IM, Kondo Y, Kondo S. Arsenic trioxide induces autophagic cell death in malignant glioma cells by upregulation of mitochondrial cell death protein BNIP3. *Oncogene* (2005) 24(6):980–91. doi: 10.1038/sj.onc.1208095
186. Kim EH, Sohn S, Kwon HJ, Kim SU, Kim MJ, Lee SJ, et al. Sodium selenite induces superoxide-mediated mitochondrial damage and subsequent autophagic cell death in malignant glioma cells. *Cancer Res* (2007) 67 (13):6314–24. doi: 10.1158/0008-5472.can-06-4217
187. Torres S, Lorente M, Rodríguez-Fornés F, Hernández-Tiedra S, Salazar M, García-Taboada E, et al. A combined preclinical therapy of cannabinoids and temozolomide against glioma. *Mol Cancer Ther* (2011) 10(1):90–103. doi: 10.1158/1535-7163.mct-10-0688
188. Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. *Cell Death Different* (2004) 11(4):448–57. doi: 10.1038/sj.cdd.4401359
189. Sotelo J, Briceño E, López-González MA. Adding chloroquine to conventional treatment for glioblastoma multiforme: a randomized, double-blind, placebo-controlled trial. *Ann Internal Med* (2006) 144 (5):337–43. doi: 10.7326/0003-4819-144-5-200603070-00008
190. Rosenfeld MR, Ye X, Supko JG, Desideri S, Grossman SA, Brem S, et al. A phase I/II trial of hydroxychloroquine in conjunction with radiation therapy and concurrent and adjuvant temozolomide in patients with newly diagnosed glioblastoma multiforme. *Autophagy* (2014) 10(8):1359–68. doi: 10.4161/auto.28984
191. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) 144(5):646–74. doi: 10.1016/j.cell.2011.02.013
192. Masui K, Cavenee WK, Mischel PS. mTORC2 and Metabolic Reprogramming in GBM: at the Interface of Genetics and Environment. *Brain Pathol (Zurich Switzerland)* (2015) 25(6):755–9. doi: 10.1111/bpa.12307
193. Zhou W, Wahl DR. Metabolic Abnormalities in Glioblastoma and Metabolic Strategies to Overcome Treatment Resistance. *Cancers (Basel)* (2019) 11 (9):1231. doi: 10.3390/cancers11091231
194. Albrecht LV, Bui MH, De Robertis EM. Canonical Wnt is inhibited by targeting one-carbon metabolism through methotrexate or methionine deprivation. *Proc Natl Acad Sci* (2019) 116(8):2987–95. doi: 10.1073/pnas.1820161116

195. Ducker GS, Rabinowitz JD. One-Carbon Metabolism in Health and Disease. *Cell Metab* (2017) 25(1):27–42. doi: 10.1016/j.cmet.2016.08.009
196. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* (2012) 2(5):401–4. doi: 10.1158/2159-8290.CD-12-0095
197. Kryukov GV, Wilson FH, Ruth JR, Paulk J, Tsherniak A, Marlow SE, et al. MTAP deletion confers enhanced dependency on the PRMT5 arginine methyltransferase in cancer cells. *Science* (2016) 351(6278):1214–8. doi: 10.1126/science.aad5214
198. Marjon K, Cameron MJ, Quang P, Clasquin MF, Mandley E, Kunii K, et al. MTAP Deletions in Cancer Create Vulnerability to Targeting of the MAT2A/PRMT5/RIOK1 Axis. *Cell Rep* (2016) 15(3):574–87. doi: 10.1016/j.celrep.2016.03.043
199. Mavrakis KJ, McDonald ER. 3rd, Schlabach MR, Billy E, Hoffman GR, deWeck A, et al. Disordered methionine metabolism in MTAP/CDKN2A-deleted cancers leads to dependence on PRMT5. *Science* (2016) 351 (6278):1208–13. doi: 10.1126/science.aad5944
200. Banasavadi-Siddegowda YK, Welker AM, An M, Yang X, Zhou W, Shi G, et al. PRMT5 as a druggable target for glioblastoma therapy. *Neuro-oncology* (2018) 20(6):753–63. doi: 10.1093/neuonc/nox206
201. Tasset I, Cuervo AM. Role of chaperone-mediated autophagy in metabolism. *FEBS J* (2016) 283(13):2403–13. doi: 10.1111/febs.13677
202. Schneider JL, Suh Y, Cuervo AM. Deficient chaperone-mediated autophagy in liver leads to metabolic dysregulation. *Cell Metab* (2014) 20(3):417–32. doi: 10.1016/j.cmet.2014.06.009
203. Lv L, Li D, Zhao D, Lin R, Chu Y, Zhang H, et al. Acetylation targets the M2 isoform of pyruvate kinase for degradation through chaperone-mediated autophagy and promotes tumor growth. *Mol Cell* (2011) 42(6):719–30. doi: 10.1016/j.molcel.2011.04.025
204. Xia HG, Najafov A, Geng J, Galan-Acosta L, Han X, Guo Y, et al. Degradation of HK2 by chaperone-mediated autophagy promotes metabolic catastrophe and cell death. *J Cell Biol* (2015) 210(5):705–16. doi: 10.1083/jcb.201503044
205. Masui K, Tanaka K, Akhavan D, Babic I, Gini B, Matsutani T, et al. mTOR complex 2 controls glycolytic metabolism in glioblastoma through FoxO acetylation and upregulation of c-Myc. *Cell Metab* (2013) 18(5):726–39. doi: 10.1016/j.cmet.2013.09.013
206. Suwala AK, Hanaford A, Kahlert UD, Maciaczyk J. Clipping the Wings of Glioblastoma: Modulation of WNT as a Novel Therapeutic Strategy. *J Neuropathol Exp Neurol* (2016) 75(5):388–96. doi: 10.1093/jnen/nlw013
207. Suwala AK, Koch K, Rios DH, Aretz P, Uhlmann C, Ogorek I, et al. Inhibition of Wnt/beta-catenin signaling downregulates expression of aldehyde dehydrogenase isoform 3A1 (ALDH3A1) to reduce resistance against temozolomide in glioblastoma in vitro. *Oncotarget* (2018) 9 (32):22703–16. doi: 10.18632/oncotarget.25210
208. Odia Y, Iwamoto FM, Moustakas A, Fraum TJ, Salgado CA, Li A, et al. A phase II trial of enzastaurin (LY317615) in combination with bevacizumab in adults with recurrent malignant gliomas. *J Neuro-Oncol* (2016) 127(1):127–35. doi: 10.1007/s11060-015-2020-x
209. Kurokawa C, Geekiyanage H, Allen C, Iankov I, Schroeder M, Carlson B, et al. Alisertib demonstrates significant antitumor activity in bevacizumab resistant, patient derived orthotopic models of glioblastoma. *J Neuro-Oncol* (2017) 131(1):41–8. doi: 10.1007/s11060-016-2285-8
210. Song A, Andrews DW, Werner-Wasik M, Kim L, Glass J, Bar-Ad V, et al. Phase I trial of alisertib with concurrent fractionated stereotactic re-irradiation for recurrent high grade gliomas. *Radiother Oncol: J Eur Soc Ther Radiol Oncol* (2019) 132:135–41. doi: 10.1016/j.radonc.2018.12.019
211. Escamilla-Ramírez A, Castillo-Rodríguez RA. Autophagy as a Potential Therapy for Malignant Glioma. *Pharmaceuticals (Basel)* (2020) 13(7):156. doi: 10.3390/ph13070156

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Coelho, Fernandes, Boccacino, Souza, Melo-Escobar, Alves, Prado, Iglesia, Cangiano, Mazzaro and Lopes. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Oncology

REVIEW
published: 25 November 2020
doi: 10.3389/fonc.2020.606436

Emerging Autophagy Functions Shape the Tumor Microenvironment and Play a Role in Cancer Progression - Implications for Cancer Therapy

Silvina Odete Bustos, Fernanda Antunes, Maria Cristina Rangel and Roger Chammas**

Instituto do Cancer do Estado de São Paulo, Faculdade de Medicina de São Paulo, Brazil

The tumor microenvironment (TME) is a complex environment where cancer cells reside and interact with different types of cells, secreted factors, and the extracellular matrix. Additionally, TME is shaped by several processes, such as autophagy. Autophagy has emerged as a conserved intracellular degradation pathway for clearance of damaged organelles or aberrant proteins. With its central role, autophagy maintains the cellular homeostasis and orchestrates stress responses, playing opposite roles in tumorigenesis. During tumor development, autophagy also mediates autophagy-independent functions associated with several hallmarks of cancer, and therefore exerting several effects on tumor suppression and/or tumor promotion mechanisms. Beyond the concept of degradation, new different forms of autophagy have been described as modulators of cancer progression, such as secretory autophagy enabling intercellular communication in the TME by cargo release. In this context, the synthesis of senescence-associated secretory proteins by autophagy lead to a senescent phenotype. Besides disturbing tumor treatment responses, autophagy also participates in innate and adaptive immune signaling. Furthermore, recent studies have indicated intricate crosstalk between autophagy and the epithelial-mesenchymal transition (EMT), by which cancer cells obtain an invasive phenotype and metastatic potential. Thus, autophagy in the cancer context is far broader and complex than just a cell energy sensing mechanism. In this scenario, we will discuss the key roles of autophagy in the TME and surrounding cells, contributing to cancer development and progression/EMT. Finally, the potential intervention in autophagy processes as a strategy for cancer therapy will be addressed.

Keywords: tumor microenvironment, secretion, immune system, epithelial-mesenchymal transition, cancer, new autophagy functions

OPEN ACCESS

Edited by:
Daniel Hector Grasso, Consejo Nacional de Investigaciones Cientificas y Tecnicas (CONICET), Argentina

Reviewed by:
Daniela Laura Papademetrio, University of Buenos Aires, Argentina
Clarissa Ribeiro Reily Rocha, Federal University of São Paulo, Brazil

****Correspondence:***
Silvina Bustos
silvinabvg@gmail.com
Roger Chammas
rchammas@usp.br

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *14 September 2020*
Accepted: *22 October 2020*
Published: *25 November 2020*

Citation:
Bustos SO, Antunes F, Rangel MC and Chammas R (2020) Emerging Autophagy Functions Shape the Tumor Microenvironment and Play a Role in Cancer Progression - Implications for Cancer Therapy. Front. Oncol. 10:606436. doi: 10.3389/fonc.2020.606436

INTRODUCTION

The autophagy process has been explored for almost 60 years, from morphological studies since early 70's to molecular studies initiated in the 1990s (1–3). During this period, several studies were conducted to understand the genetic mechanisms of autophagy, leading to the discovery of autophagy-related genes (ATG) in yeast (4, 5). Subsequently, ATG homologs were identified in various organisms, and new ATG genes were described in mammals (6). Building upon these findings, efforts to delve into the molecular mechanisms involved in the degradation of intracellular constituents have grown rapidly. However, several issues remain unsolved regarding the molecular regulation of autophagy, its integration and control at the tissue and systemic levels and its role in cancer pathophysiology. Three main types of autophagy have been described, depending on the morphology and mechanisms: microautophagy, chaperone-mediated autophagy and the best characterized macroautophagy (hereafter referred as autophagy).

Autophagy is an evolutionarily conserved process responsible for removing intracellular molecular aggregates of misfolded proteins and damaged organelles, through the sequestration of these substrates in a double-membrane vesicle, which fuses with lysosomes, where degradation of the macromolecular machines or complexes takes place. Multiple proteins are involved in the sequential stages of autophagy consisting in initiation, elongation of isolated membranes decorated by microtubule-associated protein 1A/1B-light chain 3 (LC3) to form an autophagosome, and its fusion with the lysosome to cargo degradation. Autophagy is stimulated in physiological and pathological conditions regulating cell metabolism and homeostasis (7). In cancer cells, stressors, such as hypoxia or nutrient deprivation, induce autophagy to support the high energy demand of cells with dysregulated proliferation. Many tumor suppressor and oncogene products are elements of autophagy pathways, pointing to the relationship between autophagy and tumorigenesis. Moreover, it is well known that many cancer cells have high basal levels of autophagy. Although autophagy contributes to cancer promotion in advanced stages, it is also capable to inhibit tumor initiation in early stages (8, 9). The molecular circuitry controlling autophagy is therefore complex, as it can either induce cell-death or promote cell survival (**Figure 1**) (10, 11). Understanding the mechanisms for the protective role of autophagy in cancer is essential for the identification of novel targets to the control of resistance of tumors to treatment.

The process of recycling cellular components performed by autophagy has been well characterized. Beyond self-eating and recycling damaged organelles, new roles for autophagy and the ATG genes have been ascribed (12, 13). Indeed, autophagy interferes in a wide range of cellular processes. Interestingly, components of autophagy can influence dynamic cellular processes and lead to tumor microenvironment (TME) reprogramming. Here, we discuss the novel roles of the autophagy machinery in tumor secretion, immune response, migration, and invasion capacity of tumor cells undergoing the epithelial to mesenchymal transition (EMT) (14, 15). These processes may occur simultaneously or not, affecting not only tumor cells, but also tumor microenvironmental components. These processes may also be interconnected and thus interfering with tumor progression, amplifying the roles of autophagy in tumor development and heterogeneity.

AUTOPHAGY AS A MECHANISM OF PROTEIN SECRETION

Among the diverse functions triggered by autophagy, "autophagy secretion" has received attention for its ability to alter the

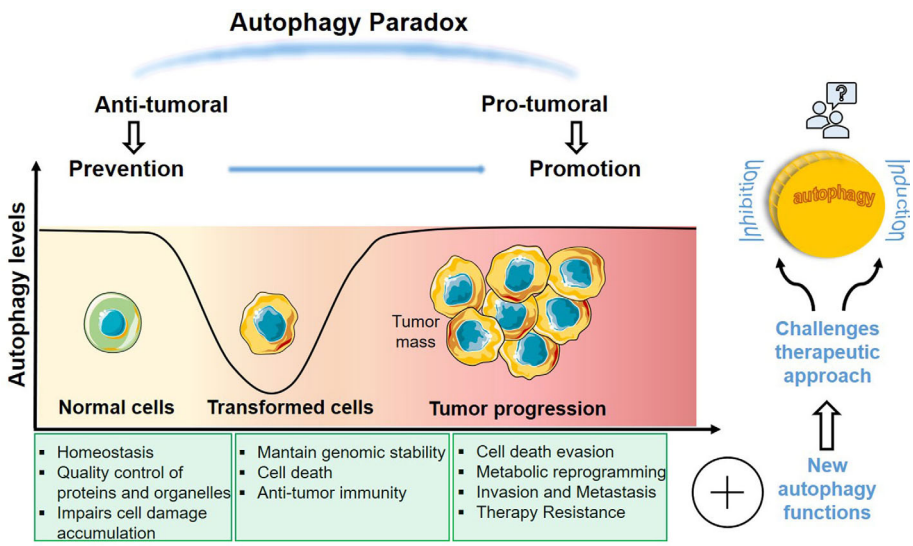


FIGURE 1 | Dual role of autophagy in cancer. Autophagy is implicated in several stages of tumorigenesis executing different processes. The extensive and opposite functions in cancer makes autophagy an important target to develop new therapies. A deeper knowledge about this complex feature of autophagy in cancer research is essential to find more accurate therapeutic approaches.

secretory profile of the tumor microenvironment, participating in the modulation of processes related to cancer progression (16, 17). Since the term autophagy secretion does not culminate in degradation into lysosomes, we adopt here, like some authors, the term autophagy-dependent secretion (ADS) (18). Nowadays, it is well established that some components of the autophagy route are involved in both conventional and unconventional secretion pathways (19). The conventional secretion route is the best-studied route for protein secretion; it can be regulated positively and negatively by autophagy components, for instance during protein recycling or through the selective clearance of secretory vesicles (20, 21). Unlike proteins exported through the conventional secretory pathway, the cargo delivered to the extracellular space or the plasma membrane by unconventional secretion (UPS) lacks the specific signal peptide and bypasses the classical Endoplasmic Reticulum (ER)-to-Golgi pathways of protein secretion (22). Usually, this pathway is activated by cellular stress, being an alternative route to proteins that use the conventional secretion (23).

Over the last few years, various studies have shown that autophagy takes part in the secretion of several proteins that critically contribute to tumor development. Among them are included different types of cargo, such as High Mobility Group Box 1 (HMGB1), IL1-β and other cytokines, immune mediators, and RNAs (18, 24, 25). Although the major complexes of classical autophagy and their molecular machinery have been clarified, novel and specific autophagy-dependent processes are still under investigation. Despite that, some evidence about the mechanism of intracellular traffic in ADS has emerged by the examination of unconventional secretion in yeast and the alternative route of IL1-β extracellular secretion (**Table 1**) (26, 49). Thereby, the results revealed that some markers involved in this pathway are shared with the classical autophagy program, but there are others exclusive to ADS. Findings of the machinery implicated in cargo selection and release have been suggested three different pathways.

First, the ATG genes stimulate the generation of an intermediate membrane, not a regular autophagosome, required to LC3I lipidation (LC3II) and the cargo contained within the inner membrane is subsequently delivered extracellularly instead to the lysosomes (50). Second, leaderless proteins are translocated to the intermembrane space of an autophagosome and released directly by fusion with the plasma membrane or with multivesicular bodies (MVBs). The last process proposed consists of an MVB/amphisome intermediate (late endosome-MVB and autophagosome fusion) and the secretion of material in small extracellular vesicles (51).

Considering all strategies, recent studies have been shown that the ADS needs SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) proteins, as SEC22B, to prevent the fusion with lysosomes and drive the select target to the plasma membrane (52). Besides the regulation by ATG proteins, for instance, ATG5/ATG12/ATG16L1 complex and ATG3, the unconventional secretion also requires of cargo receptors as TRIM (Tripartite motif family) proteins, specifically TRIM16, as well as LC3, GRASP65 (Golgi reassembly-stacking protein)and the GTPase Rab8A of the Ras family, necessary for sorting the target to the plasma membrane

TABLE 1 | Evidence summary of studies related to molecular mechanisms and components of autophagy implicated in the three topics covered in this review: secretion, epithelial to mesenchymal transition (EMT) and immunity.

Process	Interfacing mechanisms	References
Autophagy-dependent secretion	• TASCC is Rag guanosine triphosphatase–dependent, necessary to recruit mTOR and favor protein secretion. • Atg9L and LC3 cooperate to expand the protein secretion compartment. GRASP, Sec, Atg5 and Vsp proteins are required in the process. • Specialized SNAREs, as Sec22b and receptors, like TRIM16, coordinate secretory autophagy. ESCRT components also are involved in autophagy secretion pathway.	Narita et al. (20) Duran et al. (26) Dupont et al. (16) Gee et al. (23) Kimura et al. (27) Noh et al. (28) Rabouille (29)
Epithelial - Mesenchymal Transition	• TGFβ activates autophagy in early phases of cancer progression; in later phases inhibits ULK1 promoting EMT. Autophagy attenuates EMT by degradation of SNAIL, TWIST and SLUG and activation of ROS-NFκB-HIF1α pathway. • ROS-NFκB-HIF1α pathway stimulates SNAIL, N-cadherin expression and thus EMT. • Autophagy increase HMGB1 expression and TGF-β/Smad3 signaling enhancing EMT markers. • Beclin-1 signaling inhibits EMT by down- regulation of WNT1, ZEB1, and NF-κB. Accelerates EMT increasing Twist and Vimentin.	Gugnoni et al. (30) Zi et al. (31) Wang et al. (32) Li et al. (33) Bao et al. (34) Li et al. (35) Cicchini et al. (36) Catalano et al. (37)
Immunity	• LAP: ULK and Rab7 independent. Activated by membrane receptors like TLR2. Dependent of Rubicon and NADPH oxidase (NOX2) to produce ROS and recruit LC3. • T cell function involves autophagy activation by TCR or IL-2 receptor, SQSTM1/p62 and Bcl10 degradation. Autophagy controls T homeostasis due expression of ATG3, Vps34, ATG7, ATG5. • MHC antigen presentation: Dribbles formation *via* SQSTM1/p62 and LC3. • Macrophages differentiation: Involves beclin-1 release from BCL-2 and ATG5 cleavage. • Immune cells differentiation and function regulation.	Heckmann et al. (38) Botbol et al. (39) Murera et al. (40) Merkley et al. (41) Munz (42) Zhang et al. (43) Pua et al. (44) Xu et al. (45) Salio et al. (46) Clark and Simon (47) Sil et al. (48)

(27, 53). Curiously, proteins implicated in extracellular vesicle secretion, like VCP and Rab7, were also found in autophagy pathways and conversely, key ATG proteins, such as ATG12/3 and ATG5, were identified as crucial regulators of exosome biogenesis (54–57). Thus, this data demonstrated a potential link between autophagy and extracellular vesicles (EVs) machinery, processes that contribute to cellular communication and signaling in the tumor microenvironment acting as modulators of tumor progression and aggressiveness. All data collected until now expose the relevance of the set of proteins released by ADS to contribute with some of the hallmarks of cancer (58).

Most of the proteins released by ADS can activate invasion and metastasis, induce resistance to cancer therapies and/or promote inflammation, helping tumor cells to mitigate stress. Such set includes cytokines that use unconventional routes, as TGF-β and IL1-β, both responsible for playing antagonistic roles within the tumor microenvironment, depending on the cellular context (59, 60). Despite that, many studies support their functions as tumor promotors influencing in the inflammatory response of immune cells and contributing to immune suppression, tumor growth, angiogenesis, and metastasis, as observed in breast cancer cells, where the secretion of IL1-β drives colonization of the bone microenvironment, establishing a metastatic niche and cell proliferation (61, 62).

Similar responses were observed in HMGB1 and ATP secretion. Extracellular HMGB1 induces pro-inflammatory cytokines and regulates other genes leading to cell migration and metastasis. HMGB1exerts pro-tumoral functions favoring prostate cancer cells survival and cancer progression (63). Simultaneously, HMGB1 is also responsible for autophagy induction. Regarding ATP, recent studies support its role in tumor survival by switching the ATP-gated receptor P2x to nfP2X$_7$, a non-pore functional, that impairs the membrane permeability and the subsequent cell death (64). In addition, IL-6 and IL-8 secreted by autophagy are key determinants of the senescence-associated secretion phenotype (SASP), characteristic of senescence activated by DNA damage-mediated signals (65–67). High levels of both cytokines secreted by tumor cells and other cells such as cancer-associated fibroblasts (CAF) establish a senescent microenvironment and increase tumor aggressiveness showing a correlation with cancer progression and poor prognosis in many tumor types (68, 69). For example, in colorectal cancer, it was showed that peritoneal mesothelial cells control metastasis in SW480 cells and stimulate proliferation by the activation of senescence along with the secretion of mediators as IL-6 and IL-8 (70). Based on these findings and the prominent role of autophagy in cancer, various researchers have hypothesized a link between autophagy and senescence induction (71).

The Dual Interface of Autophagy and Senescence

One of the first evidence of autophagy acting as an effector mechanism of senescence came from Young et al. (12), who demonstrated, in an oncogene-induced senescence model (OIS), that up-regulation of ATG genes induced autophagy and senescence, while the inhibition of *ATG7* and *ATG5* by shRNA delayed senescence. The OIS program is a dynamic process consisting of an initial phase of rapid proliferation and mTOR activation, a transition phase where diverse changes in morphology, signaling, translation and mTOR activity occur, culminating in a senescence phase, achieved by diverse senescence programs. Thus, autophagy is activated by stress, oncogenic stress, helping to shift the proliferative cell state to a senescent state through the fast protein remodeling and the synthesis/secretion of proteins as IL-6 and IL-8. Later, the same group demonstrated that autophagy is involved in IL-6, IL-8 secretion in a posttranslational manner since the mRNA levels remain stable in ATG knockdown cells. Secretion of these cytokines was further associated with a new type of autophagy called TOR- autophagy spatial coupling compartment (TASCC), which is located at the trans side of Golgi apparatus of senescent cells to accumulate autolysosomes, and mTOR1 facilitating the biosynthesis and secretion of proteins (20, 72). These secretion events were related to survival in tumor cells dependent on autophagy (73, 74). Moreover, several studies in different cell types endorsed the connection between these processes, but the mechanisms are not completely understood and occasionally contradictory, making it crucial to assess what type of autophagy program has been activated (75, 76). Collectively, there is evidence supporting pro-senescence and anti-senescence mechanisms induced by autophagy, including those promoting senescence under different conditions (77, 78).

As a pro-senescence program, a set of studies of Caparelli et al. (79–81), validated an autophagy-senescence transition (AST) process which consists of autophagy activation, metabolism alteration and the subsequent senescence induction in CAFs, responsible to promote tumor growth. They also showed that overexpression of CDK inhibitors (p16/p19/p21) was able to induce autophagy and senescence in CAFs and breast cancer cells favoring tumor promotion. Another study illustrated the notion that p53-mediated senescence is regulated by autophagy, which leads to the degradation of a p53 isoform capable of inhibiting the whole protein, and thereby inducing senescence (82). Likewise, the loss of p53 function can boost SASP in cells and promote tumor growth (83). However, the induction of senescence by wild type p53 has also been reported in different cellular contexts (84, 85). In a different approach, Knizhnik, and collaborators demonstrated that temozolomide triggers autophagy in glioma cells through the generation of DNA adducts, leading to senescence and not apoptosis, thus playing a role in cell survival rather than cell death (86). Besides, exposure of cancer cells to either chemotherapeutic agents or irradiation-induced autophagy is followed by cellular senescence. The entry to senescence has been described as a tumor suppressor mechanism limiting the replication of premalignant cells (75, 87). Although therapy-induced senescence has the intent to suppress cancer cell growth, senescent cells can also contribute with the survival of non-damaged neighboring cells. This protumoral effect of senescence, a bystander effect by SASP activation, may consequently stimulate invasion and tumor progression (88). Alternatively, studies in human fibroblasts

showed that autophagy impairment by ATG7, ATG12 knockdown induces premature senescence mediated by activation of p53 and the generation of reactive oxidative species from dysfunctional mitochondria (89). In line with this, a work using a glioma model driven by oncogenic *KRAS* observed that autophagy inhibition using *KRAS:shAtg7* cells predisposes cell to senescence, characterized by β-galactosidase activity and SASP markers (90). A similar outcome came from data of miR-212 in prostate cancer. Interestingly, the authors found that miR-212 is upregulated in benign regions compared with PCa tissues and responsible to negatively modulate autophagy, inducing premature senescence by inhibiting SIRT1. Thus, miR-212 controls senescence induction, acting as a tumor suppressor (91).

Over the last years, senescence has been considered an important process to fight cancer, encouraging the search for anti-cancer therapies based on the induction of cell senescence (92–94). However, studies based on therapy-induced senescence (TIS) indicated the emergence of adverse effects on cancer treatment (95, 96). Chemotherapy-induced SASP drives bone loss in breast cancer and its regulation by p38-MAPK-MK2 inhibition could preserve bone, improving the quality of life of patients (97). TIS may contribute to unwanted outcomes through the stimulation of inflammation by increased secretion of SASP factors, the induction of senescence-associated stemness phenotype or senescence cell scape and further proliferation recovery (98–100). Together, these findings attracted interest to autophagy-modulated senescence and the therapeutic responses associated with both processes since senescence has been implicated with maintaining tumor dormancy, and thus mediating cancer relapse (101, 102). Then, senescence has a potential pro-tumorigenic role supporting aggressiveness, survival responses and shorter recurrence-free survival in patients (103). Finally, regarding its pro-tumorigenic role, there is increasing evidence that SASP components are involved in the establishment of an immunosuppressive environment and in the induction of EMT in TME (104–107). Further studies addressing these novel functions of autophagy and senescence in the tumor microenvironment are warranted and may pave the way to novel targeted therapies that increase the efficacy of the existing cancer treatment modalities.

The Effects of Autophagy on Epithelial-to-Mesenchymal Transition

Besides being involved in the regulation of protein secretion and tumor cell immunogenicity, autophagy has also been implicated in the process of tumor cell invasion. One of the first associations between autophagy and the invasion process was evidenced by the capacity of epithelial cells to evade anoikis *via* autophagy, what enabled cancer cells migration and invasion (108). More recently, autophagy has been connected to epithelial-to-mesenchymal transition (EMT), a critical multistep process required for cancer cells to invade and metastasize (109, 110). During EMT, epithelial cells undergo profound molecular and biochemical changes to be transiently converted into mesenchymal cells to gain motility, invasiveness, stemness characteristics, and chemoresistance. Multiple embryonic signaling pathways cooperate in the initiation and progression of EMT, including TGFβ, WNT, Hedgehog, and Notch (109, 111).

Notably, there is a multifaceted link between autophagy-correlated and EMT-correlated signaling pathways, reflected by an intricate web of regulatory signaling pathways that converge on the regulation of EMT and autophagy, and that may alter the reciprocal equilibrium between these two processes (30). These pathways often activate EMT-transcription factors and are initially triggered by extracellular signals (112). Probably, the best characterized EMT inducer is TGFβ, known to trigger EMT through the activation of SMAD, PI3K/AKT, MAPK, and Rho-GTPases (112). During cancer progression, cells that undergo EMT require autophagy activation to survive the metastatic spreading. On the other hand, autophagy tends to inhibit the early phases of metastasis, contrasting the activation of the EMT mainly by selectively destabilizing crucial mediators of this process, such as TGFβ (**Table 1**). As part of the tumor-suppressive program dependent of TGFβ, it would promote autophagy in the early phases of tumor formation. On the other hand, later in tumor progression, TGFβ would restrain autophagy while inducing EMT and promoting metastatic spreading of cancer cells (30). Regarding TGFβ and the convergence of signaling pathways between both processes, it was identified recently that the autophagic activity mediated by the transcription factor EB regulates TGFβ signaling in melanoma. Blockage of the BRAFi-induced autophagy function led to an augment of EMT activation and metastasis by enhancing TGFβ signaling, which was responsible for driving tumor progression (113).

Based on the complex relationship between autophagy and EMT, controversies have emerged in the literature regarding the role of autophagy inhibition on EMT: while several studies implicate autophagy in the promotion of EMT, others have suggested the inverse, indicating that inhibition of autophagy could promote EMT and consequently induce cancer cell invasion. Although considerable evidence suggests that the inhibition of autophagy will improve cancer therapy and despite early phase clinical trials show promising results for the use of hydroxychloroquine for this purpose (114), others have highlighted possible undesirable effects of the inhibition of autophagy in cancer therapy (31, 115, 116). Supporting the beneficial effect of autophagy inhibition during cancer progression, there are several compounds and/or microenvironmental conditions that activate the EMT program, and can also induce an autophagic response in different types of cultured cancer and non-cancerous cells, impairing EMT. It has been suggested that EMT impairment could benefit the treatment efficacy of renal cell carcinoma with existing therapeutic regimen when combined to the autophagy inhibitor chloroquine, supporting the evidence that an EMT-like phenotype corresponds to a higher autophagic flux (15). In this regard, it has been also suggested that autophagy is required for EMT induction and metastasis in hepatoblastoma cells (117) and for TGFβ1-induced EMT in non-small-cell lung carcinoma cells (118). Additionally, autophagy induced by starvation was able to activate migration, invasion, and EMT marker expression upon

rapamycin induction, and BECN1 knockdown reverted this phenotype. (119). Also, following mTOR signaling inhibition, which is known to induce autophagy, the migration, invasion and EMT marker expression were reduced in colorectal cancer cells (120). Moreover, autophagy is critical for hepatocellular carcinoma cells invasion through the induction of EMT and activation of TGF-β/Smad3-dependent signaling, which plays a key role in regulating autophagy-induced EMT (33), as well as is required for TGFβ2-induced EMT and reactive oxygen species (ROS) modulation in these cells (34). Another model of hepatocellular carcinoma revealed that inhibition of autophagy did not alter cell migration, invasion or EMT marker expression *in vitro*, however sensitized cells to anoikis and decreased lung metastases *in vivo* (121). Therefore, the role of autophagy in EMT seems context-dependent and indicates that the effects of autophagy inhibition in the establishment of metastasis are not necessarily due to its effects on EMT, but rather on its effects on other steps of the metastatic process or in the promotion of cell death. In this regard, autophagic stimulation of metastasis could be simply a consequence of its pro-survival activity against the apoptotic signals coming from changes in adhesion and cytoskeleton reorganization (121).

Ultimate evidence has indicated that autophagy activation could rather induce a reversion of the EMT phenotype and several anticancer compounds that induce autophagy also inhibit EMT (**Table 2**) (37, 122–129, 135). By its dual role in cancer, the effect of autophagy on EMT appears controversial and likely dependent on the cellular type and/or stage of tumor progression (130–134). Thus, at early stages of metastasis, autophagy could inhibit the EMT program mainly by destabilizing EMT crucial players. Later, metastatic cells could require sustained autophagy to survive environmental and metabolic stressful conditions encountered (30). Therefore, our efforts should be concentrated in selecting the precise approaches needed to stimulate or block autophagy in a time/context-dependent manner, to primarily suppress EMT and control cancer progression.

Autophagy Plays an Essential Role in Metastatic Dormancy

Once the transformation has occurred, autophagy can maintain cellular senescence to avoid the proliferation of transformed cells (109). Accumulating evidence indicates that autophagy is also a fundamental characteristic of stem cells, including cancer stem cells (CSCs). As CSCs are likely to play a central role in tumor dormancy, it appears that autophagy could contribute to the capacity of these cells to survive for extended periods of time in a dormant state and eventually give rise to recurrent tumors that are determinants of morbidity and mortality in cancer patients. Hence, once a tumor is established, tumor cells use autophagy as a survival mechanism to metabolic stress and hypoxia, to maintain tumor-related inflammation, CSCs survival and resistance to therapy.

The 'reawakening' of tumor cells at distant sites leading to the outgrowth of metastatic disease many years after primary tumors were treated has led to the concept of metastatic dormancy (136–138). Several studies have shown a role for autophagy in promoting cancer cells survival during dormancy (139). Autophagy may promote the dormancy of disseminated tumor cells simply by supplying key amino acids and other nutrients or, autophagy may play a more instructive role by eliminating mitochondria, modulating redox balance, and actively promoting the CSC state (136, 140, 141). However, it has been suggested that dormant tumor cells are CSCs that depend upon autophagy to survive at distant sites over extended periods of time to expand later as metastatic lesions composed of both CSCs and non-CSCs, representing the full heterogeneity of rapidly growing tumors (136, 139). Indeed, dormant disseminated cancer cells can survive for several years before recurring as extremely aggressive metastatic tumors. There are relevant observations providing insights into the connection between autophagy and dormancy. Despite the autophagy-associated dormancy has not been fully elucidated and some results seem

TABLE 2 | Examples of autophagy modulation and its role in epithelial-mesenchymal transition (EMT) in cancer.

Autophagy	Cell/tissue	Function	References
Induction by the overexpression of DEDD (death effector domain-containing protein)	Breast cancer tissues	EMT inhibition	Lv et al. (122)
Induction by AZD2014 (mTOR inhibitor)	Hepatocelullar carcinoma	EMT inhibition	Liao (123)
Induction by Alisertib (Aurora Kinase A inhibitor)	Osteosarcoma Colorectar cancer cells	EMT inhibition	Niu (124) Ren (125)
Induction by Metformin (AMPK activation)	Thyroid cancer	EMT inhibition	Han et al. (126)
Induction by Danusertib (Aurora Kinase A/B/C inhibitor)	Ovarian cancer	EMT inhibition	Zi (31)
Induction by Rapamycin or PP242 (mTORi)	Glioblastoma cells	Reverse EMT, inhibit invasion	Catalano et al. (37) Mecca et al. (127)
Induction by Brusatol (Nrf2 inhibition)	Hepatocelullar carcinoma	Suppress invasion capacity and EMT	Ye et al. (128)
Induction by SB202190 and SP600125 (p38-JNKi)	Ovarian cancer cells	EMT inhibition	Chen et al. (129)
Inhibition by cloroquine	Hepatocarcinoma cells	EMT inhibition	Hu et al. (130)
Inhibition by Cudraxanthone D	Oral squamous cell carcinoma	Suppress EMT	Yu et al. (131)
Induction by Rapamycin	Acidic gastric cancer cells	Antimetastatic effect, Reverse EMT	Wang et al. (132)
Induction by Cisplatin (DNA damage)	Nasopharyngeal carcinoma cells	Promote EMT	Su et al. (133)
Induction by Alteronol (Akt/mTORi)	Melanoma cells	Promote invasion	Bao et al. (134)

controversial or related to specific phenomena, several studies recognize its crosstalk with cancer relapse (142). Findings in dormant breast cancer cells support that autophagy is crucial to promote their metastasis and survival, probably preventing the accumulation of ROS and damaged organelles (143). Additionally, increased unfolded protein response (UPR) markers have been found in dormant cells. Considering the established link between autophagy and UPR under stress conditions, it has been suggested that UPR-induced autophagy activation in dormant cells to sustain tumor survival (144, 145). Breast cancer stem cells (BCSCs), which undergo tumor initiation and unlimited self-renewal, also exhibit dormancy-associated phenotypes by upregulating autophagy during metastatic dormancy to survive environmental stress and nutrient poor conditions. Consequently, therapeutic targeting of autophagy is actively being pursued as an attractive strategy to alleviate metastatic disease and the recurrence of dormant BCSCs (146).

In conclusion, dormant cancer cells are especially dependent on autophagy for survival, which provides a rationale for combining autophagy inhibition with conventional therapeutic strategies to eliminate these cells and prevent subsequent metastatic outgrowth (147).

Autophagy and Immune System

As mentioned before, autophagy has an important role in innate and adaptative immunity and can act in several steps of the immune response, leading to its activation or inhibition, depending the context, taking part in tumor immunosurveillance. Besides the ability to modulate the TME through its secretory function, autophagy regulates cellular components (natural killer (NK) cells, dendritic cells (DC), macrophages and lymphocytes (T and B cells)) of immune response, acting on differentiation, proliferation, activation, survival and homeostasis of these cells (**Table 1**). Moreover, autophagy acts on cytokines (interleukins (IL), interferons (IFN), transforming growth factors (TGF)) and antibodies production as well as phagocytosis. Interestingly, cytokines can act as autophagy stimulators or autophagy inhibitors (148).

Autophagy also has a role in tumor response to immunotherapy and a better understand of autophagy-modulation of innate and adaptative immune response could contribute to better strategies to circumvent immunotherapy resistance. For example, autophagy enhances antigen delivery to immune cells (antigen-presenting cells (APCs) and CD8+ cytotoxic T lymphocytes) and in this way can initiate an immune response against tumor cells and enhance immunotherapy efficacy. However, in the case of cancer development, autophagy is a double edge sword for immunity since it can inhibit immune response and attenuate immunotherapy outcomes (149, 150).

Autophagy and Innate Immunity

Innate immunity is the first defense of eukaryotic cells against invading pathogens and autophagy participates in the process with autophagy adaptor proteins that interact with pattern recognition receptors and activates immune response together with elimination of intracellular invaders (47). The activation of innate immune receptors as Toll-like receptors (TLRs) and nucleotide oligomerization domain-like receptors (NLRs) induces innate-immunity-mediated autophagy upregulation (150–152). TLRs interact with pathogens on the cell surface and usually are also expressed in cancer cells, inducing cytokines production together with NF-κB and MAPK pathways activation (151–153). TLRs are proposed to activate autophagy as demonstrated for TLR2 that enhances innate immunity through ERK and JNK signaling pathways after autophagy stimulation, and also boosts autophagy in glioma cells being correlated with poorer patients outcome (154, 155). For TLR4, the autophagy induction is mediated by TRIF (Toll-IL-1 receptor adapter-inducing IFN)/RIP1 (receptor-interacting protein)/p38-MAPK axis (156). TLR4 and TLR3 activation after LPS (lipopolysaccharides) treatment induces autophagy by TRIF pathway, which contributes to TRAF6 ubiquitination followed by MAPK and NF- κB activation and harmful cytokine production, leading to lung cancer cell migration and invasion (157). In p62 *knockout* cancer cells, stimulation of TLR4 induced activation of the TRAF6-BECN1-autophagy axis leads to cancer cell migration and invasion (158). Additionally, in patients with luminal breast cancer, higher levels of TLR4 and accumulation of LC3II were observed in CAFs. These features were associated with a more aggressive relapse and poorer prognosis in the cohort of patients studied (159). Taken together, TLR and autophagy activation can contribute to tumor development since it enhances survival and proliferation of cancer cells and also triggers the release of cytokines and immunosuppressive factors, contributing to immune evasion and tumor cell resistance (160).

NLR family members, such as NOD1 and NOD2, that recognize intracytoplasmic pathogens, can also activate NF-κB and MAPK pathways and produce immunosuppressive cytokines, as well as induce autophagy by recruiting ATG16L1 (161, 162). Both NOD1 and NOD2, altering the balance of anti- and pro-inflammatory cytokines, can modulate the risk of cancer development (163). For example, in triple negative breast cancer (TNBC), the expression of NOD1 and NOD2 is associated with cancer progression and a global proteome profiling of TNBC-derived cells overexpressing these receptors demonstrated disrupted immune-related pathways such as NF- κB and MAPK signaling and autophagy (164).

Autophagy and Adaptive Immunity

Autophagy participates in adaptative immune response such as thymus selection, lymphocyte development and homeostasis, antigen presentation and cytokine release, exerting anti-tumor effects (47, 165). Adaptative immunity occurs when extracellular or intracellular peptide epitopes are presented by APCs through the major histocompatibility complex (MHC) class I and II to CD8+ and CD4+ T cells, respectively. The interaction of antigen and T cell receptors triggers cellular (cytotoxic lymphocytes) and humoral (antibody-producing B cells) adaptative immune response (42, 166). The efficient antigen presentation requires proteasomal or lysosomal antigen degradation and delivery of resulting peptides to MHC molecules and this step can be enhanced by autophagy, for example, in APCs upon uptake of extracellular antigens (e.g. tumor antigens) and in antigen processing for MHC I cross- presentation. Autophagosomes facilitate intracellular trafficking of these antigens

to endosomes to be degraded by cathepsins followed by peptide load onto MHC II molecules that mature and get translocated to the plasma membrane and present antigens to CD4+ T cells (42, 166). A non-canonical regulation of phagocytosis by ATG proteins can also be used to engulfment of extracellular antigens, which is known as LC3-associated phagocytosis (LAP), characterized by a single membrane vesicle decorated with LC3-II instead of double-membrane autophagosome as in autophagy (**Table 1**) (42, 167).

Autophagy and Immune Cells

Autophagy has multiple roles on immune cells acting during their differentiation, proliferation, activation, and homeostasis maintenance and in this setting can also promote or inhibit tumor development (150). Dendritic cells link the innate and adaptative immune system as they are powerful professional APCs. Autophagy is involved in different DC functions both in physiological and pathological conditions (168). The inhibition of autophagy impacts the ability of DCs to process and present cytoplasmic antigens through the MHC II pathway and cytokines secretion, which increases their immunostimulatory phenotype (169–171). Macrophages are also APCs that require autophagy during the differentiation process in monocytes from the bone marrow into macrophages in tissue site (43, 172). Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a signal to maturation and prevents monocytes apoptosis together with autophagy induction. When autophagy is downregulated either by BECN1 knockdown or pharmacological inhibition using 3-methyladenine (3-MA) and chloroquine, caspases are activated and cytokine production is prevented (43). Autophagy is also involved in macrophage polarization with its inhibition leading to the classical activation profile and augmented pro-inflammatory cytokines secretion, and its induction promoting macrophage alternative activation, resulting in increased production of anti-inflammatory cytokines (173).

T cells use basal autophagy to maintain organelle homeostasis and it can be induced after T cell antigen receptor (TCR) stimulation. Moreover impaired autophagy after deletion of ATG proteins (ATG3, ATG5, and ATG7), BECN1 or Vps34 can hinder T cell survival, proliferation, differentiation, and activation (150, 174–176). Autophagy proteins may also be involved in other functions besides autophagy as demonstrated for memory CD8+ T cell, in which UVRAG (ultraviolet radiation resistance-associated protein) deletion does not impair autophagy but affects proliferation (177). On CD4+ T cells, autophagy impairment after BECN1 deletion leads to apoptosis upon TCR stimulation (178). On the other hand, blockage of mTOR signaling after rapamycin treatment in effector CD8+ T cells can enhance memory CD8+ T cells in lymphoid tissue or inhibit them in mucosal tissue (179). In antigen-specific memory CD8+ T cells, deficient autophagy leads to the accumulation of damaged mitochondria and increased apoptosis (180). Moreover, mTOR status can also interfere with T cell differentiation since its induction lead to activated T cell to differentiate into Th cells and its downregulation together with AMPK induction cause naïve T cells differentiation into regulatory T (Treg) cells (181). The metabolic profile also influences the dependence on autophagy since cells as memory lymphocytes and Treg cells, that use more oxidative phosphorylation (OXPHOS), are more dependent on autophagy for homeostasis than effector T cells that use preferentially aerobic glycolysis (45, 181, 182). On Treg cells, impaired autophagy induces mTORC1 and MYC signaling pathways, contributing to apoptosis induction (182).

For B cells, autophagy participates during cell development to support extremely high metabolic demands for their differentiation and reaches maximal levels during the earliest stages of development and diminish as B cells mature. It is also important, at basal levels, to maintain peripheral B cell numbers as required to cell survival after LPS stimulation, as well as for IgM production after immunization. However, autophagy is not essential for transition of pro- to pre-B cell stages in the bone marrow and B cell activation after BCR stimulation (183). Mature B cells with impaired autophagy (Atg5-/-) accumulate damaged organelles and have enlarged endoplasmic reticulum together with ER stress, more antibody secretion and plasma cells apoptosis (184).

Autophagy is also required for NK cell differentiation, since it regulates the number and quality of mitochondria on proliferating NK cells and enhances memory NK cells in an ATG3 dependent manner (185). In invariant natural killer cells (iNKT), it is observed a high level of autophagy during iNKT cell thymic differentiation into memory cells to regulate mitochondrial content and ROS production. A conditional deletion of Atg7 gene in T-cell compartment blocked iNKT development and maturation, as well augmented its susceptibility to apoptosis (46). In another study, the deletion of Atg5 or Atg7 decreased iNKT mature cells and IL-4 and IFN-γ levels accompanied by an increase in apoptosis (186).

In neutrophils, autophagy deficiency has no impact on their morphology, migration, granular content, apoptosis or effector functions, but in autophagy-deficient mice, neutrophil proliferation and differentiation is augmented, indicating an inverse correlation between autophagy and neutrophil differentiation (187).

As mentioned previously, there are feedback loops between autophagy and different cytokines. For IL-1 (IL-1α and IL-1β), autophagy limits its secretion although it is observed autophagy induction by these cytokines, indicating a negative feedback mechanism (188, 189). The interferon family (IFN types I and II) also induces autophagy in epithelial, immune, and tumor cells (190, 191). IL-2, IL-12, and TGF-β also stimulate autophagy (192–194). On the other hand, IL-6 has an anti-autophagic effect in starvation-induced autophagy in U937 cells (195) but stimulates autophagy in B cells (196). IL-10 also inhibits starvation-induced autophagy in DCs (197).

Autophagy can also act in immune tolerance mediated by immunotherapy strategies since immunologic molecules such as indoleamine 2,3-dioxygenase (IDO), PD-1 and CTLA-4 can be regulated by autophagy pathways. IDO is found in tumor sites and has anti-tumor immunity effects through interference with a cytotoxic T-cell response, DC maturation and increase in Treg population, promoting immunologic tolerance and tumor development, but its production can be inhibited by autophagy stimulation (198, 199). PD-1 from tumor cell surface interacts with PD-L1 on T-cells and acts as an inhibitory checkpoint molecule, preventing recognition of tumor cells, suppressing T

cell proliferation, development, and anti-tumor immunity. It has been reported that the interaction of PD1 with its ligand limits nutrients availability to nearby T-cells, promoting autophagy induction (200). Treatment with Sigma1 can induce autophagy in co-cultured T-cells and tumor cells, leading to degradation of PD-L1 and suppression of PD-1 and PD-L1 interaction, which could favor immunotherapy effects due to immune microenvironment modulation (201). However, the expression of PD-L1 affects several genes involved in mTOR signaling and autophagy. When PD-L1 is lost, it is observed autophagy upregulation and less sensitization to autophagy inhibitors to reduce tumor cell proliferation (202). CTLA-4 is another immune tolerance checkpoint and an effective target for tumor treatment. In human melanomas, over-expression of MAGE-A, a cancer-germline antigen, is associated with CTLA-4 blockade resistance and can downregulate autophagy, suggesting autophagy induction as a potential therapeutic approach to improve CTLA-4 inhibitors efficacy (203).

Autophagy in Immune Cells: Dual Functions Shaping Tumor Response

Increasing data suggest that autophagy can interfere with antitumor immunity together with tumor development and survival (149, 150). Knockdown of ATG5 in cancer cells was followed by increased induction of DC maturation, production of IL-6 and IFN-γ along with the proliferation of CD4+ and CD8+ T-cells after an immunogenic cell death inducer treatment (204). In Treg cells, autophagy is an important and active process to support their homeostasis contributing to their immunosuppressive profile. Suboptimal NK cell activity induces autophagy in surviving tumor cells, leading to treatment resistance (205). As mentioned before, many pro-inflammatory cytokines contribute to tumor growth, metastasis and can induce autophagy (206). Although the treatment with high-dose-IL-2 has antitumor effects it is limited by severe side effects, as multiorgan dysfunction that is accompanied by systemic autophagic syndrome induced by cytokines. In a murine model of metastatic liver tumor, the combined treatment of high-dose-IL2 and chloroquine increased antitumor effects along with decreased toxicity, increased long-term survival and enhanced infiltration of immune cells in liver (207). In a renal cell carcinoma model, autophagy inhibition also improved HDIL-2 anti-tumor effects due to apoptosis induction and immune system stimulation together with increased activity of DCs, T-cells, and NK cells (208). Inhibition of autophagy through 3-MA treatment also potentiates apoptosis induced by IL-24 in oral squamous cell carcinomas, demonstrating that autophagy inhibition can be explored as a promising approach to increase immunotherapy efficacy (209). The phytochemical shikonin can induce necroptosis accompanied by autophagy enhancement that directly contributes to DAMP upregulation in tumor cells. However, if autophagy flux is blocked by chloroquine treatment, there is an even greater upregulation of ectoDAMPS, resulting in DC activation. In the context of DC vaccines, the pretreatment of tumor cells with chloroquine and shikonin potentiated antimetastatic activity and reduced chemotherapy doses *in vivo* (210). Autophagy can also reduce immunotherapy effect by impairing cytotoxic T-lymphocyte (CTL)-mediated tumor cell lysis when autophagy is induced under hypoxia conditions, activating STAT3 signaling in target cells which in turn favors tumor cell survival, proliferation, and immune escape. If autophagy is blocked in this context and CTL-response is boosted with a vaccination strategy, vaccination efficacy is improved, leading to tumor regression *in vivo* (211). Hypoxia-induced autophagy also impairs NK cells function by degradation of NK-derived granzyme B in autophagosomes of hypoxic breast cancer cells, leading them less susceptible to NK killing and immunotherapy effects. However, if autophagy is blocked by deletion of BECN1, granzyme B levels are restored and favors tumor regression *in vivo* due to tumor cell death by NK-mediated lysis (212). Pancreatic ductal adenocarcinoma (PDAC) is known for immune checkpoint blockade resistance and frequently altered MHC-I expression that facilitates immune evasion trough NBR1 selective autophagy downregulation of MHC-I. If autophagy is inhibited, there is recover in MHC-I expression and augmented immunotherapy response along with enhanced T-cell immunity in tumor models *in vivo* (213). In a cohort of gastric cancer (GC) patients, the expression of CXCL10 has a positive correlation with patient prognosis and induces T lymphocyte migration and infiltration into the GC 3D cell culture model. It is also observed in basal conditions that GC cells have increased autophagy, and the knockdown of essential autophagy genes (Atg5 and Atg7) or their pharmacological block in these cells augmented CXCL10 expression under normal and hypoxic conditions facilitating T cell lymphocyte migration and potentiating tumor immunity (214). Recent studies demonstrated that autophagy activation in tumor cells is one of the main reasons for decreased antitumor immune response, reinforcing the concept of autophagy inhibition as a valuable approach to increase immunotherapy results. One of the autophagic proteins that have been recently described as drug targetable is Vps34, whose inhibition with genetic or pharmacological approaches decreased tumor growth along with increased mice survival due to infiltration of immune cells (NK, CD8+ and CD4+ T effector cells) within the tumor microenvironment, which could turn cold tumors into hot inflamed tumors to enable immunotherapy treatments. Moreover, the combined treatment of Vps34 inhibitor and anti-PD-L1/PD-1 in melanoma and colorectal cancer models prolonged mice survival and enhanced immunotherapy benefits (215). Impairment of autophagy with BECN1 ablation is also beneficial to increase NK infiltration and inhibit tumor growth in a melanoma tumor model. In addition, NK infiltration in the tumor microenvironment is mediated by CCL5 chemokine overexpression in autophagy-deficient cells trough c-Jun/JNK activation. Similar results were also obtained after deletion of other autophagic genes as *Atg5* and SQSTM1/p62 and pharmacological inhibition by chloroquine. In conclusion, targeting autophagy may be a valuable approach to improve immunotherapy mediated by NK cells (216).

On the other hand, autophagy has also anti-tumoral effects since its induction contributes to a better response of helper T

lymphocytes (HTLs) against head and neck squamous carcinoma cells and its inhibition decreases HTLs recognition of tumor cells (217). Tumors can act as antigen donor cells that require autophagy to form tumor-derived autophagosomes (Dribbles) which contain tumor-associated antigens. Dribbles can stimulate efficient cross-presentation of T-cells (218) and induce B cell activation along with cytokine release and antibody production (219) which can contribute to tumor control and elimination. For vaccination strategies, in contrast to whole-cell tumor vaccine, Dribbles prime T cells by enhancing costimulatory molecules as well as MHCI, and reduce tumor formation on hosts challenged with nonhomologous tumors, effect limited if there is depletion of the autophagic protein SQSTM1/p62 (220, 221). For efficient immunotherapy, tumor antigens should be immunogenic, essential only for tumor cells and overexpressed in tumors compared to normal tissue. Recently, it was demonstrated that SQSTM1/p62 fits all these requisites and a DNA-vaccine encoding this protein had antitumor and antimetastatic effect against several tumor models in dogs, suggesting that this can be a useful strategy for immunotherapy (222). Inactivation of ATG5 in non-small cell lung carcinoma favors carcinogenesis and its development is accelerated in KRASAtg5(fl/fl) autophagy-deficient mice. In these mice, a higher expression of ENTPD1/CD39 was observed, culminating in an immunosuppressive environment along with increased Treg infiltration that contributes to tumor development (223). As discussed previously, autophagy plays a role in monocytes/macrophages recruitment, what decreases infiltration in liver tissues accompanied by autophagy reduction and hepatocarcinogenesis (224). Immunotherapy can also be potentiated by autophagy. For instance, in murine tumor models, the treatment with chemotherapy or radiotherapy induced autophagy, which favored translocation of the mannose-6-

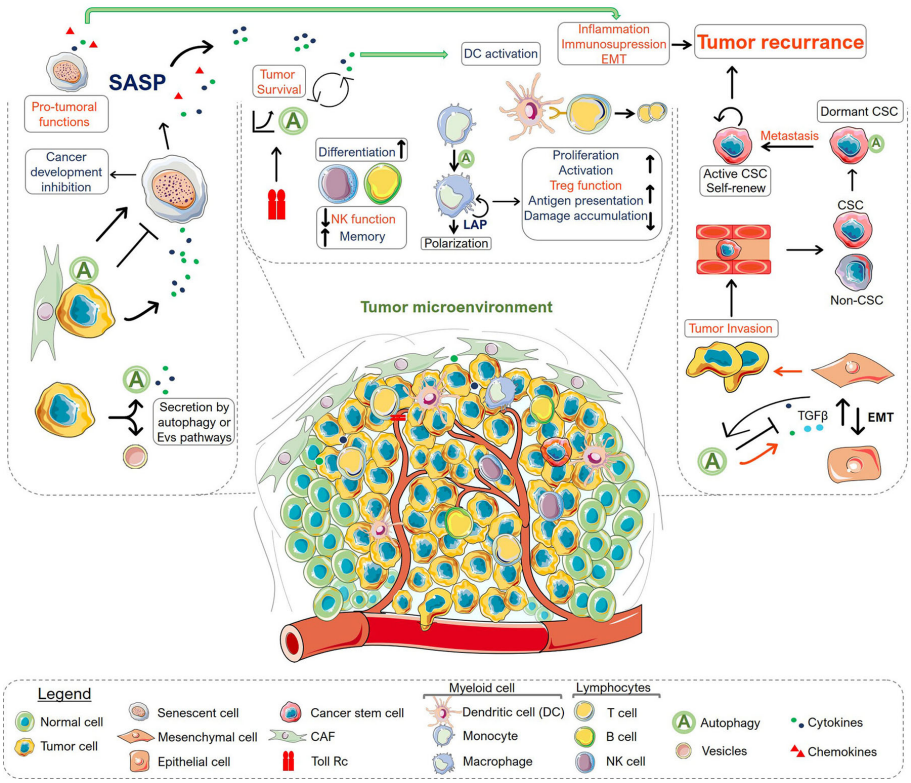


FIGURE 2 | Overview of autophagy roles in the tumor microenvironment. The scheme summarizes the role of autophagy in secretion (left), immune system (middle), epithelial-mesenchymal transition (EMT) and tumor dormancy (right). There is an intricate and dynamic network of signaling circuits that drive tumor development and progression within the tumor microenvironment. The connectivity among various processes may regulate the fate of the microenvironment components, indicating the importance of viewing this as an emerging system, where the resulting interactions are larger than the sum of the individual parcels. Autophagy can act in many ways in different types of cells displaying anti-tumoral (shown in blue) or pro-tumoral functions (shown in red). Protein secretion by CAF or tumor cells can modulate cellular states inducing or inhibiting senescence, which ultimately can control tumor survival, immune cell response and interfere with the epithelial-mesenchymal transition, affecting tumor invasion capacity. In the context of the immune system, autophagy has a key role in immune cell differentiation, proliferation, activation and effector function, covering the range of homeostatic to reactive functions of the immune system. At the same time, autophagy is also connected with the innate immune response being controlled by receptors such as TLRs. Importantly, in advanced stages, the autophagy system in tumor cells is involved with EMT and the consequent ability of cancer cells to invade tissues and metastasize. The interplay among these functions contributes to tumor aggressiveness. Moreover, autophagy was also appointed as a characteristic of cancer stem cells (CSC) playing a central role in tumor dormancy. Altogether, the myriad of connected process regulated by autophagy in the TME modulate tumor response and may determine its regression or progression. Altogether, understanding the integrated mechanisms that regulate autophagy within the TME constitute a niche for development of novel strategies for combination therapy.

phosphate receptor (MPR) from autophagosomes to tumor cells surface, rendering the cells more sensitive to granzyme B from activated CTLs, potentiating CTLs killing and immunotherapy (225, 226). In another example, autophagy elicited by alpha-tocopheryloxyacetic acid (α-TEA) on tumor cells improved cross-presentation of tumor antigens for MHCI and MHCII, which can be use as an adjuvant strategy to improve anti-tumor immune responses and strength immunotherapy (227, 228). Treatment of ovarian cancer models with farletuzumab, a humanized monoclonal antibody against folate receptor α, also induced autophagy and reduced proliferation, which was reversed by autophagy pharmacological blockage (229). Finally, studies indicate that although statins have no protective effect on breast cancer incidence, they can be used as adjuvant therapy to increase apoptosis and radio-sensitivity along with proliferation and invasion inhibition of cancer cells. Fluvastatin belongs to the statin family and when used *in vitro* to treat breast cancer cells, it induced autophagy but with impaired lysosome function which may contribute to cell death. Moreover, a decrease of pro-inflammatory cytokines, such as IL-6 and TNF-α, was observed along with autophagy consequent effect in tumor immunity (230).

CONCLUDING REMARKS

Given the dual role of autophagy in cancer and its involvement in cancer therapeutic responses, the process of autophagy has been pointed as an important theme in cancer research (**Figure 1**). The interconnection of autophagy to the regulation of several biological processes in the TME indicates that autophagy has key roles in tumor progression. Thus, in addition to its primary function of degradation and recycling, most of the components of the autophagy machinery also mediate numerous non-autophagic functions. This suggests that autophagy operates in many ways, establishing an intricate network of signaling, along with other cell elements, integrating diverse signals within the TME and regulating the fate of cancer and other microenvironmental cells (**Figure 2**). Integrative approaches, which address the impact of autophagy inhibition in complex systems, are therefore necessary for the development of strategies that exploit the autophagy machinery as a target to control tumor growth, without impeding the generation of a long-lasting memory cytotoxic immune response or the induction of a stemness phenotype in residual cancer cells. Models of the intricated and dynamic network of cancer cells and the tumor microenvironmental cells are warranted for filtering compounds that may control tumor growth and increase the efficacy of many known therapeutic regimens.

AUTHOR CONTRIBUTIONS

SB, FA, MR, and RC wrote the manuscript. SB drew the figures. SB and RC designed and edited the review. All authors contributed to the article and approved the submitted version.

FUNDING

The authors' work has been funded by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 2014/03742-0 and 2015/22814-5) and Conselho de Desenvolvimento Científico e Tecnológico (CNPq 305700/2017-0).

REFERENCES

1. de Duve C, Wattiaux R. Functions of lysosomes. *Annu Rev Physiol* (1966) 28:435–92. doi: 10.1146/annurev.ph.28.030166.002251
2. Arstila AU, Trump BF. Studies on cellular autophagocytosis. The formation of autophagic vacuoles in the liver after glucagon administration. *Am J Pathol* (1968) 53(5):687–733.
3. Bolender RP, Weibel ER. A morphometric study of the removal of phenobarbital-induced membranes from hepatocytes after cessation of threatment. *J Cell Biol* (1973) 56(3):746–61. doi: 10.1083/jcb.56.3.746
4. Tsukada M, Ohsumi Y. Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae. *FEBS Lett* (1993) 333(1-2):169–74. doi: 10.1016/0014-5793(93)80398-E
5. Harding TM, Morano KA, Scott SV, Klionsky DJ. Isolation and characterization of yeast mutants in the cytoplasm to vacuole protein targeting pathway. *J Cell Biol* (1995) 131(3):591–602. doi: 10.1083/jcb.131.3.591
6. Klionsky DJ, Cregg JM, Dunn WA, Emr SD, Sakai Y, Sandoval IV, et al. A unified nomenclature for yeast autophagy-related genes. *Dev Cell* (2003) 5 (4):539–45. doi: 10.1016/S1534-5807(03)00296-X
7. Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. *Nat Rev Cancer* (2007) 7(12):961–7. doi: 10.1038/nrc2254
8. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest* (2003) 112(12):1809–20. doi: 10.1172/JCI20039
9. Liang C, Feng P, Ku B, Dotan I, Canaani D, Oh BH, et al. Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. *Nat Cell Biol* (2006) 8(7):688–99. doi: 10.1038/ncb1426
10. Corazzari M, Fimia GM, Lovat P, Piacentini M. Why is autophagy important for melanoma? Molecular mechanisms and therapeutic implications. *Semin Cancer Biol* (2013) 23(5):337–43. doi: 10.1016/j.semcancer.2013.07.001
11. Huang YH, Lei J, Yi GH, Huang FY, Li YN, Wang CC, et al. Coroglaucigenin induces senescence and autophagy in colorectal cancer cells. *Cell Prolif* (2018) 51(4):e12451. doi: 10.1111/cpr.12451
12. Young AR, Narita M, Ferreira M, Kirschner K, Sadaie M, Darot JF, et al. Autophagy mediates the mitotic senescence transition. *Genes Dev* (2009) 23 (7):798–803. doi: 10.1101/gad.519709
13. Galluzzi L, Green DR. Autophagy-Independent Functions of the Autophagy Machinery. *Cell* (2019) 177(7):1682–99. doi: 10.1016/j.cell.2019.05.026
14. Wu H, Huang S, Chen Z, Liu W, Zhou X, Zhang D. Hypoxia-induced autophagy contributes to the invasion of salivary adenoid cystic carcinoma through the HIF-1α/BNIP3 signaling pathway. *Mol Med Rep* (2015) 12 (5):6467–74. doi: 10.3892/mmr.2015.4255
15. Singla M, Bhattacharyya S. Autophagy as a potential therapeutic target during epithelial to mesenchymal transition in renal cell carcinoma: An in vitro study. *BioMed Pharmacother* (2017) 94:332–40. doi: 10.1016/j.biopha.2017.07.070
16. Dupont N, Jiang S, Pilli M, Ornatowski W, Bhattacharya D, Deretic V. Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1β. *EMBO J* (2011) 30(23):4701–11. doi: 10.1038/emboj.2011.398
17. Martins I, Wang Y, Michaud M, Ma Y, Sukkurwala AQ, Shen S, et al. Molecular mechanisms of ATP secretion during immunogenic cell death. *Cell Death Differ* (2014) 21(1):79–91. doi: 10.1038/cdd.2013.75
18. New J, Thomas SM. Autophagy-dependent secretion: mechanism, factors secreted, and disease implications. *Autophagy* (2019) 15(10):1682–93. doi: 10.1080/15548627.2019.1596479

19. Ponpuak M, Mandell MA, Kimura T, Chauhan S, Cleyrat C, Deretic V. Secretory autophagy. *Curr Opin Cell Biol* (2015) 35:106–16. doi: 10.1016/j.ceb.2015.04.016
20. Narita M, Young AR, Arakawa S, Samarajiwa SA, Nakashima T, Yoshida S, et al. Spatial coupling of mTOR and autophagy augments secretory phenotypes. *Science* (2011) 332(6032):966–70. doi: 10.1126/science.1205407
21. Lock R, Kenific CM, Leidal AM, Salas E, Debnath J. Autophagy-dependent production of secreted factors facilitates oncogenic RAS-driven invasion. *Cancer Discovery* (2014) 4(4):466–79. doi: 10.1158/2159-8290.CD-13-0841
22. Viotti C. ER to Golgi-Dependent Protein Secretion: The Conventional Pathway. *Methods Mol Biol* (2016) 1459:3–29. doi: 10.1007/978-1-4939-3804-9_1
23. Gee HY, Noh SH, Tang BL, Kim KH, Lee MG. Rescue of ΔF508-CFTR trafficking via a GRASP-dependent unconventional secretion pathway. *Cell* (2011) 146(5):746–60. doi: 10.1016/j.cell.2011.07.021
24. Cadwell K, Debnath J. Beyond self-eating: The control of nonautophagic functions and signaling pathways by autophagy-related proteins. *J Cell Biol* (2018) 217(3):813–22. doi: 10.1083/jcb.201706157
25. Rak J. L(C3)icensing of exosomes for RNA export. *Nat Cell Biol* (2020) 22 (2):137–9. doi: 10.1038/s41556-020-0466-3
26. Duran JM, Anjard C, Stefan C, Loomis WF, Malhotra V. Unconventional secretion of Acb1 is mediated by autophagosomes. *J Cell Biol* (2010) 188 (4):527–36. doi: 10.1083/jcb.200911154
27. Kimura T, Jia J, Kumar S, Choi SW, Gu Y, Mudd M, et al. Dedicated SNAREs and specialized TRIM cargo receptors mediate secretory autophagy. *EMBO J* (2017) 36(1):42–60. doi: 10.15252/embj.201695081
28. Noh SH, Gee HY, Kim Y, Piao H, Kim J, Kang CM, et al. Specific autophagy and ESCRT components participate in the unconventional secretion of CFTR. *Autophagy* (2018) 14(10):1761–78. doi: 10.1080/15548627.2018.1489479
29. Rabouille C, Malhotra V, Nickel W. Diversity in unconventional protein secretion. *J Cell Sci* (2012) 125(Pt 22):5251–5. doi: 10.1242/jcs.103630
30. Gugnoni M, Sancisi V, Gandolfi G, Manzotti G, Ragazzi M, Giordano D, et al. Cadherin-6 promotes EMT and cancer metastasis by restraining autophagy. *Oncogene* (2017) 36(5):667–77. doi: 10.1038/onc.2016.237
31. Zi D, Zhou ZW, Yang YJ, Huang L, Zhou ZL, He SM, et al. Danusertib Induces Apoptosis, Cell Cycle Arrest, and Autophagy but Inhibits Epithelial to Mesenchymal Transition Involving PI3K/Akt/mTOR Signaling Pathway in Human Ovarian Cancer Cells. *Int J Mol Sci* (2015) 16(11):27228–51. doi: 10.3390/ijms161126018
32. Wang H, Zhang C, Xu L, Zang K, Ning Z, Jiang F, et al. Bufalin suppresses hepatocellular carcinoma invasion and metastasis by targeting HIF-1α via the PI3K/AKT/mTOR pathway. *Oncotarget* (2016) 7(15):20193–208. doi: 10.18632/oncotarget.7935
33. Li J, Yang B, Zhou Q, Wu Y, Shang D, Guo Y, et al. Autophagy promotes hepatocellular carcinoma cell invasion through activation of epithelial-mesenchymal transition. *Carcinogenesis* (2013) 34(6):1343–51. doi: 10.1093/carcin/bgt063
34. Dash S, Sarashetti PM, Rajashekar B, Chowdhury R, Mukherjee S. TGF-β2-induced EMT is dampened by inhibition of autophagy and TNF-α treatment. *Oncotarget* (2018) 9(5):6433–49. doi: 10.18632/oncotarget.23942
35. Li S, Zhang HY, Du ZX, Li C, An MX, Zong ZH, et al. Induction of epithelial-mesenchymal transition (EMT) by Beclin 1 knockdown via posttranscriptional upregulation of ZEB1 in thyroid cancer cells. *Oncotarget* (2016) 7(43):70364–77. doi: 10.18632/oncotarget.12217
36. Cicchini M, Chakrabarti R, Kongara S, Price S, Nahar R, Lozy F, et al. Autophagy regulator BECN1 suppresses mammary tumorigenesis driven by WNT1 activation and following parity. *Autophagy* (2014) 10(11):2036–52. doi: 10.4161/auto.34398
37. Catalano M, D'Alessandro G, Lepore F, Corazzari M, Caldarola S, Valacca C, et al. Autophagy induction impairs migration and invasion by reversing EMT in glioblastoma cells. *Mol Oncol* (2015) 9(8):1612–25. doi: 10.1016/j.molonc.2015.04.016
38. Heckmann BL, Green DR. Correction: LC3-associated phagocytosis at a glance. *J Cell Sci* (2019) 132(5). doi: 10.1242/jcs.222984
39. Botbol Y, Guerrero-Ros I, Macian F. Key roles of autophagy in regulating T-cell function. *Eur J Immunol* (2016) 46(6):1326–34. doi: 10.1002/eji.201545955
40. Murera D, Arbogast F, Arnold J, Bouis D, Muller S, Gros F. CD4 T cell autophagy is integral to memory maintenance. *Sci Rep* (2018) 8(1):5951. doi: 10.1038/s41598-018-23993-0
41. Merkley SD, Chock CJ, Yang XO, Harris J, Castillo EF. Modulating T Cell Responses via Autophagy: The Intrinsic Influence Controlling the Function of Both Antigen-Presenting Cells and T Cells. *Front Immunol* (2018) 9:2914. doi: 10.3389/fimmu.2018.02914
42. Munz C. Autophagy proteins influence endocytosis for MHC restricted antigen presentation. *Semin Cancer Biol* (2019) 66:110–5. doi: 10.1016/j.semcancer.2019.03.005
43. Zhang Y, Morgan MJ, Chen K, Choksi S, Liu ZG. Induction of autophagy is essential for monocyte-macrophage differentiation. *Blood* (2012) 119 (12):2895–905. doi: 10.1182/blood-2011-08-372383
44. Pua HH, Dzhagalov I, Chuck M, Mizushima N, He YW. A critical role for the autophagy gene Atg5 in T cell survival and proliferation. *J Exp Med* (2007) 204(1):25–31. doi: 10.1084/jem.20061303
45. Xu X, Araki K, Li S, Han JH, Ye L, Tan WG, et al. Autophagy is essential for effector CD8(+) T cell survival and memory formation. *Nat Immunol* (2014) 15(12):1152–61. doi: 10.1038/ni.3025
46. Salio M, Puleston DJ, Mathan TS, Shepherd D, Stranks AJ, Adamopoulou E, et al. Essential role for autophagy during invariant NKT cell development. *Proc Natl Acad Sci USA* (2014) 111(52):E5678–87. doi: 10.1073/pnas.1413935112
47. Clarke AJ, Simon AK. Autophagy in the renewal, differentiation and homeostasis of immune cells. *Nat Rev Immunol* (2019) 19(3):170–83. doi: 10.1038/s41577-018-0095-2
48. Sil P, Zhao F, Muse G, Wong S-W, Kolb J, DeGraff LM, et al. Non-canonical autophagy in dendritic cells restricts cross-presentation and anti-tumor immunity. *bioRxiv* (2019) 145(5):1389–405. doi: 10.1101/789867
49. Manjithaya R, Subramani S. Role of autophagy in unconventional protein secretion. *Autophagy* (2010) 6(5):650–1. doi: 10.4161/auto.6.5.12066
50. Zhang M, Kenny SJ, Ge L, Xu K, Schekman R. Translocation of interleukin-1β into a vesicle intermediate in autophagy-mediated secretion. *Elife* (2015) 4:e11205. doi: 10.7554/eLife.11205
51. Levine B, Kroemer G. Biological Functions of Autophagy Genes: A Disease Perspective. *Cell* (2019) 176(1-2):11–42. doi: 10.1016/j.cell.2018.09.048
52. Liu Y, Flanagan JJ, Barlowe C. Sec22p export from the endoplasmic reticulum is independent of SNARE pairing. *J Biol Chem* (2004) 279 (26):27225–32. doi: 10.1074/jbc.M312122200
53. Chen YD, Fang YT, Cheng YL, Lin CF, Hsu LJ, Wang SY, et al. Exophagy of annexin A2 via RAB11, RAB8A and RAB27A in IFN-γ-stimulated lung epithelial cells. *Sci Rep* (2017) 7(1):5676. doi: 10.1038/s41598-017-06076-4
54. Galluzzi L, Baehrecke EH, Ballabio A, Boya P, Bravo-San Pedro JM, Cecconi F, et al. Molecular definitions of autophagy and related processes. *EMBO J* (2017) 36(13):1811–36. doi: 10.15252/embj.201796697
55. Kalra H, Simpson RJ, Ji H, Aikawa E, Altevogt P, Askenase P, et al. Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. *PLoS Biol* (2012) 10(12):e1001450. doi: 10.1371/journal.pbio.1001450
56. Xu J, Camfield R, Gorski SM. The interplay between exosomes and autophagy - partners in crime. *J Cell Sci* (2018) 131(15):jcs215210. doi: 10.1242/jcs.215210
57. Guo H, Chitiprolu M, Roncevic L, Javalet C, Hemming FJ, Trung MT, et al. Atg5 Disassociates the V. *Dev Cell* (2017) 43(6):716–30.e7. doi: 10.1016/j.devcel.2017.11.018
58. Singh SS, Vats S, Chia AY, Tan TZ, Deng S, Ong MS, et al. Dual role of autophagy in hallmarks of cancer. *Oncogene* (2018) 37(9):1142–58. doi: 10.1038/s41388-017-0046-6
59. Bent R, Moll L, Grabbe S, Bros M. Interleukin-1 Beta-A Friend or Foe in Malignancies? *Int J Mol Sci* (2018) 19(8):2155. doi: 10.3390/ijms19082155
60. Liu S, Chen S, Zeng J. TGF–β signaling: A complex role in tumorigenesis (Review). *Mol Med Rep* (2018) 17(1):699–704. doi: 10.3892/mmr.2017.7970
61. Fabregat I, Fernando J, Mainez J, Sancho P. TGF-beta signaling in cancer treatment. *Curr Pharm Des* (2014) 20(17):2934–47. doi: 10.2174/13816128113199990591
62. Tulotta C, Lefley DV, Freeman K, Gregory WM, Hanby AM, Heath PR, et al. Endogenous Production of IL1B by Breast Cancer Cells Drives Metastasis

and Colonization of the Bone Microenvironment. *Clin Cancer Res* (2019) 25 (9):2769–82. doi: 10.1158/1078-0432.CCR-18-2202
63. Lv DJ, Song XL, Huang B, Yu YZ, Shu FP, Wang C, et al. HMGB1 Promotes Prostate Cancer Development and Metastasis by Interacting with Brahma-Related Gene 1 and Activating the Akt Signaling Pathway. *Theranostics* (2019) 9(18):5166–82. doi: 10.7150/thno.33972
64. Gilbert SM, Oliphant CJ, Hassan S, Peille AL, Bronsert P, Falzoni S, et al. ATP in the tumour microenvironment drives expression of nfP2X. *Oncogene* (2019) 38(2):194–208. doi: 10.1038/s41388-018-0426-6
65. Cotzomi-Ortega I, Aguilar-Alonso P, Reyes-Leyva J, Maycotte P. Autophagy and Its Role in Protein Secretion: Implications for Cancer Therapy. *Mediators Inflamm* (2018) 2018:4231591. doi: 10.1155/2018/4231591
66. Coppé JP, Desprez PY, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol* (2010) 5:99–118. doi: 10.1146/annurev-pathol-121808-102144
67. Ohtani N. Deciphering the mechanism for induction of senescence-associated secretory phenotype (SASP) and its role in aging and cancer development. *J Biochem* (2019) 166(4):289–95. doi: 10.1093/jb/mvz055
68. Ortiz-Montero P, Londoño-Vallejo A, Vernot JP. Senescence-associated IL-6 and IL-8 cytokines induce a self- and cross-reinforced senescence/inflammatory milieu strengthening tumorigenic capabilities in the MCF-7 breast cancer cell line. *Cell Commun Signal* (2017) 15(1):17. doi: 10.1186/s12964-017-0172-3
69. Kim YH, Choi YW, Lee J, Soh EY, Kim JH, Park TJ. Senescent tumor cells lead the collective invasion in thyroid cancer. *Nat Commun* (2017) 8:15208. doi: 10.1038/ncomms15208
70. Mikuła-Pietrasik J, Sosińska P, Maksin K, Kucińska MG, Piotrowska H, Murias M, et al. Colorectal cancer-promoting activity of the senescent peritoneal mesothelium. *Oncotarget* (2015) 6(30):29178–95. doi: 10.18632/oncotarget.4932
71. Slobodnyuk K, Radic N, Ivanova S, Llado A, Trempolec N, Zorzano A, et al. Autophagy-induced senescence is regulated by p38α signaling. *Cell Death Dis* (2019) 10(6):376. doi: 10.1038/s41419-019-1607-0
72. Canaud G, Brooks CR, Kishi S, Taguchi K, Nishimura K, Magassa S, et al. Cyclin G1 and TASCC regulate kidney epithelial cell G. *Sci Transl Med* (2019) 11(476). doi: 10.1126/scitranslmed.aav4754
73. Maycotte P, Gearheart CM, Barnard R, Aryal S, Mulcahy Levy JM, Fosmire SP, et al. STAT3-mediated autophagy dependence identifies subtypes of breast cancer where autophagy inhibition can be efficacious. *Cancer Res* (2014) 74(9):2579–90. doi: 10.1158/0008-5472.CAN-13-3470
74. Kraya AA, Piao S, Xu X, Zhang G, Herlyn M, Gimotty P, et al. Identification of secreted proteins that reflect autophagy dynamics within tumor cells. *Autophagy* (2015) 11(1):60–74. doi: 10.4161/15548627.2014.984273
75. Kang TW, Yevsa T, Woller N, Hoenicke L, Wuestefeld T, Dauch D, et al. Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. *Nature* (2011) 479(7374):547–51. doi: 10.1038/nature10599
76. Singh K, Matsuyama S, Drazba JA, Almasan A. Autophagy-dependent senescence in response to DNA damage and chronic apoptotic stress. *Autophagy* (2012) 8(2):236–51. doi: 10.4161/auto.8.2.18600
77. Perez-Neut M, Haar L, Rao V, Santha S, Lansu K, Rana B, et al. Activation of hERG3 channel stimulates autophagy and promotes cellular senescence in melanoma. *Oncotarget* (2016) 7(16):21991–2004. doi: 10.18632/oncotarget.7831
78. Cho YY, Kim DJ, Lee HS, Jeong CH, Cho EJ, Kim MO, et al. Autophagy and cellular senescence mediated by Sox2 suppress malignancy of cancer cells. *PLoS One* (2013) 8(2):e57172. doi: 10.1371/journal.pone.0057172
79. Capparelli C, Whitaker-Menezes D, Guido C, Balliet R, Pestell TG, Howell A, et al. CTGF drives autophagy, glycolysis and senescence in cancer-associated fibroblasts via HIF1 activation, metabolically promoting tumor growth. *Cell Cycle* (2012) 11(12):2272–84. doi: 10.4161/cc.20717
80. Capparelli C, Guido C, Whitaker-Menezes D, Bonuccelli G, Balliet R, Pestell TG, et al. Autophagy and senescence in cancer-associated fibroblasts metabolically supports tumor growth and metastasis via glycolysis and ketone production. *Cell Cycle* (2012) 11(12):2285–302. doi: 10.4161/cc.20718
81. Capparelli C, Chiavarina B, Whitaker-Menezes D, Pestell TG, Pestell RG, Hulit J, et al. CDK inhibitors (p16/p19/p21) induce senescence and autophagy in cancer-associated fibroblasts, "fueling" tumor growth via paracrine interactions, without an increase in neo-angiogenesis. *Cell Cycle* (2012) 11(19):3599–610. doi: 10.4161/cc.21884
82. Horikawa I, Fujita K, Jenkins LM, Hiyoshi Y, Mondal AM, Vojtesek B, et al. Autophagic degradation of the inhibitory p53 isoform Δ133p53α as a regulatory mechanism for p53-mediated senescence. *Nat Commun* (2014) 5:4706. doi: 10.1038/ncomms5706
83. Coppé JP, Patil CK, Rodier F, Sun Y, Muñoz DP, Goldstein J, et al. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol* (2008) 6(12):2853–68. doi: 10.1371/journal.pbio.0060301
84. Zhu Y, Xu L, Zhang J, Hu X, Liu Y, Yin H, et al. Sunitinib induces cellular senescence via p53/Dec1 activation in renal cell carcinoma cells. *Cancer Sci* (2013) 104(8):1052–61. doi: 10.1111/cas.12176
85. Harajly M, Zalzali H, Nawaz Z, Ghayad SE, Ghamloush F, Basma H, et al. p53 Restoration in Induction and Maintenance of Senescence: Differential Effects in Premalignant and Malignant Tumor Cells. *Mol Cell Biol* (2016) 36 (3):438–51. doi: 10.1128/MCB.00747-15
86. Knizhnik AV, Roos WP, Nikolova T, Quiros S, Tomaszowski KH, Christmann M, et al. Survival and death strategies in glioma cells: autophagy, senescence and apoptosis triggered by a single type of temozolomide-induced DNA damage. *PLoS One* (2013) 8(1):e55665. doi: 10.1371/journal.pone.0055665
87. Braig M, Lee S, Loddenkemper C, Rudolph C, Peters AH, Schlegelberger B, et al. Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature* (2005) 436(7051):660–5. doi: 10.1038/nature03841
88. Huang YH, Yang PM, Chuah QY, Lee YJ, Hsieh YF, Peng CW, et al. Autophagy promotes radiation-induced senescence but inhibits bystander effects in human breast cancer cells. *Autophagy* (2014) 10(7):1212–28. doi: 10.4161/auto.28772
89. Kang HT, Lee KB, Kim SY, Choi HR, Park SC. Autophagy impairment induces premature senescence in primary human fibroblasts. *PLoS One* (2011) 6(8):e23367. doi: 10.1371/journal.pone.0023367
90. Gammoh N, Fraser J, Puente C, Syred HM, Kang H, Ozawa T, et al. Suppression of autophagy impedes glioblastoma development and induces senescence. *Autophagy* (2016) 12(9):1431–9. doi: 10.1080/15548627.2016.1190053
91. Ramalinga M, Roy A, Srivastava A, Bhattarai A, Harish V, Suy S, et al. MicroRNA-212 negatively regulates starvation induced autophagy in prostate cancer cells by inhibiting SIRT1 and is a modulator of angiogenesis and cellular senescence. *Oncotarget* (2015) 6(33):34446–57. doi: 10.18632/oncotarget.5920
92. Schmitt CA, Fridman JS, Yang M, Lee S, Baranov E, Hoffman RM, et al. A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. *Cell* (2002) 109(3):335–46. doi: 10.1016/S0092-8674(02)00734-1
93. Roninson IB. Tumor cell senescence in cancer treatment. *Cancer Res* (2003) 63(11):2705–15.
94. Nardella C, Clohessy JG, Alimonti A, Pandolfi PP. Pro-senescence therapy for cancer treatment. *Nat Rev Cancer* (2011) 11(7):503–11. doi: 10.1038/nrc3057
95. Saleh T, Tyutynuk-Massey L, Cudjoe EK, Idowu MO, Landry JW, Gewirtz DA. Non-Cell Autonomous Effects of the Senescence-Associated Secretory Phenotype in Cancer Therapy. *Front Oncol* (2018) 8:164. doi: 10.3389/fonc.2018.00164
96. Patel NH, Sohal SS, Manjili MH, Harrell JC, Gewirtz DA. The Roles of Autophagy and Senescence in the Tumor Cell Response to Radiation. *Radiat Res* (2020) 194(autophagy and senescence):103–15. doi: 10.1667/RADE-20-00009
97. Yao Z, Murali B, Ren Q, Luo X, Faget DV, Cole T, et al. Therapy-Induced Senescence Drives Bone Loss. *Cancer Res* (2020) 80(5):1171–82. doi: 10.1158/0008-5472.CAN-19-2348
98. Demaria M, O'Leary MN, Chang J, Shao L, Liu S, Alimirah F, et al. Cellular Senescence Promotes Adverse Effects of Chemotherapy and Cancer Relapse. *Cancer Discovery* (2017) 7(2):165–76. doi: 10.1158/2159-8290.CD-16-0241
99. Milanovic M, Fan DNY, Belenki D, Däbritz JHM, Zhao Z, Yu Y, et al. Senescence-associated reprogramming promotes cancer stemness. *Nature* (2018) 553(7686):96–100. doi: 10.1038/nature25167
100. Saleh T, Tyutyunyk-Massey L, Murray GF, Alotaibi MR, Kawale AS, Elsayed Z, et al. Tumor cell escape from therapy-induced senescence. *Biochem Pharmacol* (2019) 162:202–12. doi: 10.1016/j.bcp.2018.12.013
101. Mosieniak G, Sliwinska MA, Alster O, Strzeszewska A, Sunderland P, Piechota M, et al. Polyploidy Formation in Doxorubicin-Treated Cancer

Cells Can Favor Escape from Senescence. *Neoplasia* (2015) 17(12):882–93. doi: 10.1016/j.neo.2015.11.008
102. Wang B, Kohli J, Demaria M. Senescent Cells in Cancer Therapy: Friends or Foes? *Trends Cancer* (2020) 6(10):838–57. doi: 10.1016/j.trecan.2020.05.004
103. Eggert T, Wolter K, Ji J, Ma C, Yevsa T, Klotz S, et al. Distinct Functions of Senescence-Associated Immune Responses in Liver Tumor Surveillance and Tumor Progression. *Cancer Cell* (2016) 30(4):533–47. doi: 10.1016/j.ccell.2016.09.003
104. Ruhland MK, Loza AJ, Capietto AH, Luo X, Knolhoff BL, Flanagan KC, et al. Stromal senescence establishes an immunosuppressive microenvironment that drives tumorigenesis. *Nat Commun* (2016) 7:11762. doi: 10.1038/ncomms11762
105. Canino C, Mori F, Cambria A, Diamantini A, Germoni S, Alessandrini G, et al. SASP mediates chemoresistance and tumor-initiating-activity of mesothelioma cells. *Oncogene* (2012) 31(26):3148–63. doi: 10.1038/onc.2011.485
106. Goulet CR, Champagne A, Bernard G, Vandal D, Chabaud S, Pouliot F, et al. Cancer-associated fibroblasts induce epithelial-mesenchymal transition of bladder cancer cells through paracrine IL-6 signalling. *BMC Cancer* (2019) 19(1):137. doi: 10.1186/s12885-019-5353-6
107. Miao JW, Liu LJ, Huang J. Interleukin-6-induced epithelial-mesenchymal transition through signal transducer and activator of transcription 3 in human cervical carcinoma. *Int J Oncol* (2014) 45(1):165–76. doi: 10.3892/ijo.2014.2422
108. Fung C, Lock R, Gao S, Salas E, Debnath J. Induction of autophagy during extracellular matrix detachment promotes cell survival. *Mol Biol Cell* (2008) 19(3):797–806. doi: 10.1091/mbc.e07-10-1092
109. Rojas-Sanchez G, Cotzomi-Ortega I, Pazos-Salazar NG, Reyes-Leyva J, Maycotte P. Autophagy and Its Relationship to Epithelial to Mesenchymal Transition: When Autophagy Inhibition for Cancer Therapy Turns Counterproductive. *Biol (Basel)* (2019) 8(4):71. doi: 10.3390/biology8040071
110. Marcucci F, Ghezzi P, Rumio C. The role of autophagy in the cross-talk between epithelial-mesenchymal transitioned tumor cells and cancer stem-like cells. *Mol Cancer* (2017) 16(1):3. doi: 10.1186/s12943-016-0573-8
111. Chen HT, Liu H, Mao MJ, Tan Y, Mo XQ, Meng XJ, et al. Crosstalk between autophagy and epithelial-mesenchymal transition and its application in cancer therapy. *Mol Cancer* (2019) 18(1):101. doi: 10.1186/s12943-019-1030-2
112. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol* (2014) 15(3):178–96. doi: 10.1038/nrm3758
113. Li S, Song Y, Quach C, Guo H, Jang GB, Maazi H, et al. Transcriptional regulation of autophagy-lysosomal function in BRAF-driven melanoma progression and chemoresistance. *Nat Commun* (2019) 10(1):1693. doi: 10.1038/s41467-019-09634-8
114. Amaravadi RK, Kimmelman AC, Debnath J. Targeting Autophagy in Cancer: Recent Advances and Future Directions. *Cancer Discovery* (2019) 9(9):1167–81. doi: 10.1158/2159-8290.CD-19-0292
115. Subramani R, Gonzalez E, Arumugam A, Nandy S, Gonzalez V, Medel J, et al. Nimbolide inhibits pancreatic cancer growth and metastasis through ROS-mediated apoptosis and inhibition of epithelial-to-mesenchymal transition. *Sci Rep* (2016) 6:19819. doi: 10.1038/srep19819
116. Kocaturk NM, Akkoc Y, Kig C, Bayraktar O, Gozuacik D, Kutlu O. Autophagy as a molecular target for cancer treatment. *Eur J Pharm Sci* (2019) 134:116–37. doi: 10.1016/j.ejps.2019.04.011
117. Chen C, Liang QY, Chen HK, Wu PF, Feng ZY, Ma XM, et al. DRAM1 regulates the migration and invasion of hepatoblastoma cells via autophagy-EMT pathway. *Oncol Lett* (2018) 16(2):2427–33. doi: 10.3892/ol.2018.8937
118. Alizadeh J, Glogowska A, Thliveris J, Kalantari F, Shojaei S, Hombach-Klonisch S, et al. Autophagy modulates transforming growth factor beta 1 induced epithelial to mesenchymal transition in non-small cell lung cancer cells. *Biochim Biophys Acta Mol Cell Res* (2018) 1865(5):749–68. doi: 10.1016/j.bbamcr.2018.02.007
119. Shen H, Yin L, Deng G, Guo C, Han Y, Li Y, et al. Knockdown of Beclin-1 impairs epithelial-mesenchymal transition of colon cancer cells. *J Cell Biochem* (2018) 119(8):7022–31. doi: 10.1002/jcb.26912
120. Gulhati P, Bowen KA, Liu J, Stevens PD, Rychahou PG, Chen M, et al. mTORC1 and mTORC2 regulate EMT, motility, and metastasis of colorectal cancer via RhoA and Rac1 signaling pathways. *Cancer Res* (2011) 71(9):3246–56. doi: 10.1158/0008-5472.CAN-10-4058
121. Peng YF, Shi YH, Ding ZB, Ke AW, Gu CY, Hui B, et al. Autophagy inhibition suppresses pulmonary metastasis of HCC in mice via impairing anoikis resistance and colonization of HCC cells. *Autophagy* (2013) 9(12):2056–68. doi: 10.4161/auto.26398
122. Lv Q, Wang W, Xue J, Hua F, Mu R, Lin H, et al. DEDD interacts with PI3KC3 to activate autophagy and attenuate epithelial-mesenchymal transition in human breast cancer. *Cancer Res* (2012) 72(13):3238–50. doi: 10.1158/0008-5472.CAN-11-3832
123. Liao H, Huang Y, Guo B, Liang B, Liu X, Ou H, et al. Dramatic antitumor effects of the dual mTORC1 and mTORC2 inhibitor AZD2014 in hepatocellular carcinoma. *Am J Cancer Res* (2015) 5(1):125–39.
124. Niu NK, Wang ZL, Pan ST, Ding HQ, Au GH, He ZX, et al. Pro-apoptotic and pro-autophagic effects of the Aurora kinase A inhibitor alisertib (MLN8237) on human osteosarcoma U-2 OS and MG-63 cells through the activation of mitochondria-mediated pathway and inhibition of p38 MAPK/PI3K/Akt/mTOR signaling pathway. *Drug Des Devel Ther* (2015) 9:1555–84. doi: 10.2147/DDDT.S74197
125. Ren BJ, Zhou ZW, Zhu DJ, Ju YL, Wu JH, Ouyang MZ, et al. Alisertib Induces Cell Cycle Arrest, Apoptosis, Autophagy and Suppresses EMT in HT29 and Caco-2 Cells. *Int J Mol Sci* (2015) 17(1):41. doi: 10.3390/ijms17010041
126. Han B, Cui H, Kang L, Zhang X, Jin Z, Lu L, et al. Metformin inhibits thyroid cancer cell growth, migration, and EMT through the mTOR pathway. *Tumour Biol* (2015) 36(8):6295–304. doi: 10.1007/s13277-015-3315-4
127. Mecca C, Giambanco I, Donato R, Arcuri C. Targeting mTOR in Glioblastoma: Rationale and Preclinical/Clinical Evidence. *Dis Markers* (2018) 2018:9230479. doi: 10.1155/2018/9230479
128. Ye R, Dai N, He Q, Guo P, Xiang Y, Zhang Q, et al. Comprehensive anti-tumor effect of Brusatol through inhibition of cell viability and promotion of apoptosis caused by autophagy via the PI3K/Akt/mTOR pathway in hepatocellular carcinoma. *BioMed Pharmacother* (2018) 105:962–73. doi: 10.1016/j.biopha.2018.06.065
129. Chen X, Chen Y, Lin X, Su S, Hou X, Zhang Q, et al. The Drug Combination of SB202190 and SP600125 Significantly Inhibit the Growth and Metastasis of Olaparib-resistant Ovarian Cancer Cell. *Curr Pharm Biotechnol* (2018) 19(6):506–13. doi: 10.2174/1389201019666180713102656
130. Hu S, Wang L, Zhang X, Wu Y, Yang J, Li J. Autophagy induces transforming growth factor-β-dependent epithelial-mesenchymal transition in hepatocarcinoma cells through cAMP response element binding signalling. *J Cell Mol Med* (2018) 22(11):5518–32. doi: 10.1111/jcmm.13825
131. Yu SB, Kang HM, Park DB, Park BS, Kim IR. Cudraxanthone D Regulates Epithelial-Mesenchymal Transition by Autophagy Inhibition in Oral Squamous Cell Carcinoma Cell Lines. *Evid Based Complement Alternat Med* (2019) 2019:5213028. doi: 10.1155/2019/5213028
132. Wang Y, Sun L, Qiu W, Qi W, Qi Y, Liu Z, et al. Inhibiting Forkhead box K1 induces autophagy to reverse epithelial-mesenchymal transition and metastasis in gastric cancer by regulating Myc-associated zinc finger protein in an acidic microenvironment. *Aging (Albany NY)* (2020) 12(7):6129–50. doi: 10.18632/aging.103013
133. Su Z, Li G, Liu C, Ren S, Deng T, Zhang S, et al. Autophagy inhibition impairs the epithelial-mesenchymal transition and enhances cisplatin sensitivity in nasopharyngeal carcinoma. *Oncol Lett* (2017) 13(6):4147–54. doi: 10.3892/ol.2017.5963
134. Bao Y, Ding Z, Zhao P, Li J, Chen P, Zheng J, et al. Autophagy inhibition potentiates the anti-EMT effects of alteronol through TGF-β/Smad3 signaling in melanoma cells. *Cell Death Dis* (2020) 11(4):223. doi: 10.1038/s41419-020-2419-y
135. Lv LH, Wan YL, Lin Y, Zhang W, Yang M, Li GL, et al. Anticancer drugs cause release of exosomes with heat shock proteins from human hepatocellular carcinoma cells that elicit effective natural killer cell antitumor responses in vitro. *J Biol Chem* (2012) 287(19):15874–85. doi: 10.1074/jbc.M112.340588
136. Sosa MS, Bragado P, Aguirre-Ghiso JA. Mechanisms of disseminated cancer cell dormancy: an awakening field. *Nat Rev Cancer* (2014) 14(9):611–22. doi: 10.1038/nrc3793
137. McGowan PM, Kirstein JM, Chambers AF. Micrometastatic disease and metastatic outgrowth: clinical issues and experimental approaches. *Future Oncol* (2009) 5(7):1083–98. doi: 10.2217/fon.09.73

138. Giancotti FG. Mechanisms governing metastatic dormancy and reactivation. *Cell* (2013) 155(4):750–64. doi: 10.1016/j.cell.2013.10.029
139. Lu Z, Luo RZ, Lu Y, Zhang X, Yu Q, Khare S, et al. The tumor suppressor gene ARHI regulates autophagy and tumor dormancy in human ovarian cancer cells. *J Clin Invest* (2008) 118(12):3917–29. doi: 10.1172/JCI35512
140. Lum JJ, Bauer DE, Kong M, Harris MH, Li C, Lindsten T, et al. Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell* (2005) 120(2):237–48. doi: 10.1016/j.cell.2004.11.046
141. Galluzzi L, Vacchelli E, Bravo-San Pedro JM, Buqué A, Senovilla L, Baracco EE, et al. Classification of current anticancer immunotherapies. *Oncotarget* (2014) 5(24):12472–508. doi: 10.18632/oncotarget.2998
142. Aqbi HF, Tyutyunyk-Massey L, Keim RC, Butler SE, Thekkudan T, Joshi S, et al. Autophagy-deficient breast cancer shows early tumor recurrence and escape from dormancy. *Oncotarget* (2018) 9(31):22113–22. doi: 10.18632/oncotarget.25197
143. Vera-Ramirez L, Vodnala SK, Nini R, Hunter KW, Green JE. Autophagy promotes the survival of dormant breast cancer cells and metastatic tumour recurrence. *Nat Commun* (2018) 9(1):1944. doi: 10.1038/s41467-018-04070-6
144. Madden E, Logue SE, Healy SJ, Manie S, Samali A. The role of the unfolded protein response in cancer progression: From oncogenesis to chemoresistance. *Biol Cell* (2019) 111(1):1–17. doi: 10.1111/boc.201800050
145. Zhang Y, Qu X, Jiang L. An oasis in the desert of cancer chemotherapeutic resistance: The enlightenment from reciprocal crosstalk between signaling pathways of UPR and autophagy in cancers. *BioMed Pharmacother* (2017) 92:972–81. doi: 10.1016/j.biopha.2017.05.132
146. La Belle Flynn A, Calhoun BC, Sharma A, Chang JC, Almasan A, Schiemann WP. Autophagy inhibition elicits emergence from metastatic dormancy by inducing and stabilizing Pfkfb3 expression. *Nat Commun* (2019) 10(1):3668. doi: 10.1038/s41467-019-11640-9
147. Mowers EE, Sharifi MN, Macleod KF. Functions of autophagy in the tumor microenvironment and cancer metastasis. *FEBS J* (2018) 285(10):1751–66. doi: 10.1111/febs.14388
148. Harris J. Autophagy and cytokines. *Cytokine* (2011) 56(2):140–4. doi: 10.1016/j.cyto.2011.08.022
149. Ishimwe N, Zhang W, Qian J, Zhang Y, Wen L. Autophagy regulation as a promising approach for improving cancer immunotherapy. *Cancer Lett* (2020) 475:34–42. doi: 10.1016/j.canlet.2020.01.034
150. Jiang GM, Tan Y, Wang H, Peng L, Chen HT, Meng XJ, et al. The relationship between autophagy and the immune system and its applications for tumor immunotherapy. *Mol Cancer* (2019) 18(1):17. doi: 10.1186/s12943-019-0944-z
151. Pan H, Chen L, Xu Y, Han W, Lou F, Fei W, et al. Autophagy-associated immune responses and cancer immunotherapy. *Oncotarget* (2016) 7 (16):21235–46. doi: 10.18632/oncotarget.6908
152. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* (2010) 11(5):373–84. doi: 10.1038/ni.1863
153. Noh JY, Yoon SR, Kim TD, Choi I, Jung H. Toll-Like Receptors in Natural Killer Cells and Their Application for Immunotherapy. *J Immunol Res* (2020) 2020:2045860. doi: 10.1155/2020/2045860
154. Lu Z, Xie D, Chen Y, Tian E, Muhammad I, Chen X, et al. TLR2 mediates autophagy through ERK signaling pathway in Mycoplasma gallisepticum-infected RAW264.7 cells. *Mol Immunol* (2017) 87:161–70. doi: 10.1016/j.molimm.2017.04.013
155. Fang L, Shen Q, Wu H, He F, Ding P, Xu K, et al. TLR2 favors OVA-induced allergic airway inflammation in mice through JNK signaling pathway with activation of autophagy. *Life Sci* (2020) 256:117896. doi: 10.1016/j.lfs.2020.117896
156. Xu Y, Jagannath C, Liu XD, Sharafkhaneh A, Kolodziejska KE, Eissa NT. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. *Immunity* (2007) 27(1):135–44. doi: 10.1016/j.immuni.2007.05.022
157. Zhan Z, Xie X, Cao H, Zhou X, Zhang XD, Fan H, et al. Autophagy facilitates TLR4- and TLR3-triggered migration and invasion of lung cancer cells through the promotion of TRAF6 ubiquitination. *Autophagy* (2014) 10 (2):257–68. doi: 10.4161/auto.27162
158. Kim MJ, Min Y, Im JS, Son J, Lee JS, Lee KY. p62 is Negatively Implicated in the TRAF6-BECN1 Signaling Axis for Autophagy Activation and Cancer Progression by Toll-Like Receptor 4 (TLR4). *Cells* (2020) 9(5). doi: 10.3390/cells9051142
159. Zhao XL, Lin Y, Jiang J, Tang Z, Yang S, Lu L, et al. High-mobility group box 1 released by autophagic cancer-associated fibroblasts maintains the stemness of luminal breast cancer cells. *J Pathol* (2017) 243(3):376–89. doi: 10.1002/path.4958
160. Braunstein MJ, Kucharczyk J, Adams S. Targeting Toll-Like Receptors for Cancer Therapy. *Target Oncol* (2018) 13(5):583–98. doi: 10.1007/s11523-018-0589-7
161. Velloso FJ, Trombetta-Lima M, Anschau V, Sogayar MC, Correa RG. NOD-like receptors: major players (and targets) in the interface between innate immunity and cancer. *Biosci Rep* (2019) 39(4). doi: 10.1042/BSR20181709
162. Travassos LH, Carneiro LA, Ramjeet M, Hussey S, Kim YG, Magalhaes JG, et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat Immunol* (2010) 11(1):55–62. doi: 10.1038/ni.1823
163. Kutikhin AG. Role of NOD1/CARD4 and NOD2/CARD15 gene polymorphisms in cancer etiology. *Hum Immunol* (2011) 72(10):955–68. doi: 10.1016/j.humimm.2011.06.003
164. Velloso FJ, Campos AR, Sogayar MC, Correa RG. Proteome profiling of triple negative breast cancer cells overexpressing NOD1 and NOD2 receptors unveils molecular signatures of malignant cell proliferation. *BMC Genomics* (2019) 20(1):152. doi: 10.1186/s12864-019-5523-6
165. Jin Y, Hong Y, Park CY, Hong Y. Molecular Interactions of Autophagy with the Immune System and Cancer. *Int J Mol Sci* (2017) 18(8):1694. doi: 10.3390/ijms18081694
166. Zhong Z, Sanchez-Lopez E, Karin M. Autophagy, Inflammation, and Immunity: A Troika Governing Cancer and Its Treatment. *Cell* (2016) 166 (2):288–98. doi: 10.1016/j.cell.2016.05.051
167. Sanjuan MA, Dillon CP, Tait SW, Moshiach S, Dorsey F, Connell S, et al. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature* (2007) 450(7173):1253–7. doi: 10.1038/nature06421
168. Ghislat G, Lawrence T. Autophagy in dendritic cells. *Cell Mol Immunol* (2018) 15(11):944–52. doi: 10.1038/cmi.2018.2
169. Liu E, Van Grol J, Subauste CS. Atg5 but not Atg7 in dendritic cells enhances IL-2 and IFN-gamma production by Toxoplasma gondii-reactive CD4+ T cells. *Microbes Infect* (2015) 17(4):275–84. doi: 10.1016/j.micinf.2014.12.008
170. Lee HK, Mattei LM, Steinberg BE, Alberts P, Lee YH, Chervonsky A, et al. In vivo requirement for Atg5 in antigen presentation by dendritic cells. *Immunity* (2010) 32(2):227–39. doi: 10.1016/j.immuni.2009.12.006
171. Tao S, Drexler I. Targeting Autophagy in Innate Immune Cells: Angel or Demon During Infection and Vaccination? *Front Immunol* (2020) 11:460. doi: 10.3389/fimmu.2020.00460
172. Jacquel A, Obba S, Solary E, Auberger P. Proper macrophagic differentiation requires both autophagy and caspase activation. *Autophagy* (2012) 8 (7):1141–3. doi: 10.4161/auto.20367
173. Liu K, Zhao E, Ilyas G, Lalazar G, Lin Y, Haseeb M, et al. Impaired macrophage autophagy increases the immune response in obese mice by promoting proinflammatory macrophage polarization. *Autophagy* (2015) 11 (2):271–84. doi: 10.1080/15548627.2015.1009787
174. Jia W, Pua HH, Li QJ, He YW. Autophagy regulates endoplasmic reticulum homeostasis and calcium mobilization in T lymphocytes. *J Immunol* (2011) 186(3):1564–74. doi: 10.4049/jimmunol.1001822
175. Pua HH, Guo J, Komatsu M, He YW. Autophagy is essential for mitochondrial clearance in mature T lymphocytes. *J Immunol* (2009) 182 (7):4046–55. doi: 10.4049/jimmunol.0801143
176. Willinger T, Flavell RA. Canonical autophagy dependent on the class III phosphoinositide-3 kinase Vps34 is required for naive T-cell homeostasis. *Proc Natl Acad Sci U S A* (2012) 109(22):8670–5. doi: 10.1073/pnas.1205305109
177. Afzal S, Hao Z, Itsumi M, Abouelkheer Y, Brenner D, Gao Y, et al. Autophagy-independent functions of UVRAG are essential for peripheral naive T-cell homeostasis. *Proc Natl Acad Sci U S A* (2015) 112(4):1119–24. doi: 10.1073/pnas.1423588112
178. Kovacs JR, Li C, Yang Q, Li G, Garcia IG, Ju S, et al. Autophagy promotes T-cell survival through degradation of proteins of the cell death machinery. *Cell Death Differ* (2012) 19(1):144–52. doi: 10.1038/cdd.2011.78
179. Sowell RT, Rogozinska M, Nelson CE, Vezys V, Marzo AL. Cutting edge: generation of effector cells that localize to mucosal tissues and form resident memory CD8 T cells is controlled by mTOR. *J Immunol* (2014) 193(5):2067–71. doi: 10.4049/jimmunol.1400074

180. Puleston DJ, Zhang H, Powell TJ, Lipina E, Sims S, Panse I, et al. Autophagy is a critical regulator of memory CD8(+) T cell formation. *Elife* (2014) 3: e03706. doi: 10.7554/eLife.03706
181. Michalek RD, Gerriets VA, Jacobs SR, Macintyre AN, MacIver NJ, Mason EF, et al. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. *J Immunol* (2011) 186(6):3299–303. doi: 10.4049/jimmunol.1003613
182. Wei J, Long L, Yang K, Guy C, Shrestha S, Chen Z, et al. Autophagy enforces functional integrity of regulatory T cells by coupling environmental cues and metabolic homeostasis. *Nat Immunol* (2016) 17(3):277–85. doi: 10.1038/ni.3365
183. Arnold J, Murera D, Arbogast F, Fauny JD, Muller S, Gros F. Autophagy is dispensable for B-cell development but essential for humoral autoimmune responses. *Cell Death Differ* (2016) 23(5):853–64. doi: 10.1038/cdd.2015.149
184. Pengo N, Scolari M, Oliva L, Milan E, Mainoldi F, Raimondi A, et al. Plasma cells require autophagy for sustainable immunoglobulin production. *Nat Immunol* (2013) 14(3):298–305. doi: 10.1038/ni.2524
185. O'Sullivan TE, Johnson LR, Kang HH, Sun JC. BNIP3- and BNIP3L-Mediated Mitophagy Promotes the Generation of Natural Killer Cell Memory. *Immunity* (2015) 43(2):331–42. doi: 10.1016/j.immuni.2015.07.012
186. Pei B, Zhao M, Miller BC, Vela JL, Bruinsma MW, Virgin HW, et al. Invariant NKT cells require autophagy to coordinate proliferation and survival signals during differentiation. *J Immunol* (2015) 194(12):5872–84. doi: 10.4049/jimmunol.1402154
187. Rozman S, Yousefi S, Oberson K, Kaufmann T, Benarafa C, Simon HU. The generation of neutrophils in the bone marrow is controlled by autophagy. *Cell Death Differ* (2015) 22(3):445–56. doi: 10.1038/cdd.2014.169
188. Harris J, Hartman M, Roche C, Zeng SG, O'Shea A, Sharp FA, et al. Autophagy controls IL-1beta secretion by targeting pro-IL-1beta for degradation. *J Biol Chem* (2011) 286(11):9587–97. doi: 10.1074/jbc.M110.202911
189. Peral de Castro C, Jones SA, Ni Cheallaigh C, Hearnden CA, Williams L, Winter J, et al. Autophagy regulates IL-23 secretion and innate T cell responses through effects on IL-1 secretion. *J Immunol* (2012) 189 (8):4144–53. doi: 10.4049/jimmunol.1201946
190. Schmeisser H, Bekisz J, Zoon KC. New function of type I IFN: induction of autophagy. *J Interferon Cytokine Res* (2014) 34(2):71–8. doi: 10.1089/jir.2013.0128
191. Matsuzawa T, Kim BH, Shenoy AR, Kamitani S, Miyake M, Macmicking JD. IFN-gamma elicits macrophage autophagy via the p38 MAPK signaling pathway. *J Immunol* (2012) 189(2):813–8. doi: 10.4049/jimmunol.1102041
192. Kang R, Tang D, Lotze MT, Zeh Iii HJ. Autophagy is required for IL-2-mediated fibroblast growth. *Exp Cell Res* (2013) 319(4):556–65. doi: 10.1016/j.yexcr.2012.11.012
193. Lin Y, Kuang W, Wu B, Xie C, Liu C, Tu Z. IL-12 induces autophagy in human breast cancer cells through AMPK and the PI3K/Akt pathway. *Mol Med Rep* (2017) 16(4):4113–8. doi: 10.3892/mmr.2017.7114
194. Zhang C, Zhang X, Xu R, Huang B, Chen AJ, Li C, et al. TGF-beta2 initiates autophagy via Smad and non-Smad pathway to promote glioma cells' invasion. *J Exp Clin Cancer Res* (2017) 36(1):162. doi: 10.1186/s13046-017-0628-8
195. Qin B, Zhou Z, He J, Yan C, Ding S. IL-6 Inhibits Starvation-induced Autophagy via the STAT3/Bcl-2 Signaling Pathway. *Sci Rep* (2015) 5:15701. doi: 10.1038/srep15701
196. Linnemann AK, Blumer J, Marasco MR, Battiola TJ, Umhoefer HM, Han JY, et al. Interleukin 6 protects pancreatic beta cells from apoptosis by stimulation of autophagy. *FASEB J* (2017) 31(9):4140–52. doi: 10.1096/fj.201700061RR
197. Martin C, Espaillat MP, Santiago-Schwarz F. IL-10 restricts dendritic cell (DC) growth at the monocyte-to-monocyte-derived DC interface by disrupting anti-apoptotic and cytoprotective autophagic molecular machinery. *Immunol Res* (2015) 63(1-3):131–43. doi: 10.1007/s12026-015-8700-y
198. Folgiero V, Miele E, Carai A, Ferretti E, Alfano V, Po A, et al. IDO1 involvement in mTOR pathway: a molecular mechanism of resistance to mTOR targeting in medulloblastoma. *Oncotarget* (2016) 7(33):52900–11. doi: 10.18632/oncotarget.9284
199. Mahoney KM, Rennert PD, Freeman GJ. Combination cancer immunotherapy and new immunomodulatory targets. *Nat Rev Drug Discovery* (2015) 14(8):561–84. doi: 10.1038/nrd4591
200. Robainas M, Otano R, Bueno S, Ait-Oudhia S. Understanding the role of PD-L1/PD1 pathway blockade and autophagy in cancer therapy. *Onco Targets Ther* (2017) 10:1803–7. doi: 10.2147/OTT.S132508
201. Maher CM, Thomas JD, Haas DA, Longen CG, Oyer HM, Tong JY, et al. Small-Molecule Sigma1 Modulator Induces Autophagic Degradation of PD-L1. *Mol Cancer Res* (2018) 16(2):243–55. doi: 10.1158/1541-7786.MCR-17-0166
202. Clark CA, Gupta HB, Sareddy G, Pandeswara S, Lao S, Yuan B, et al. Tumor-Intrinsic PD-L1 Signals Regulate Cell Growth, Pathogenesis, and Autophagy in Ovarian Cancer and Melanoma. *Cancer Res* (2016) 76(23):6964–74. doi: 10.1158/0008-5472.CAN-16-0258
203. Shukla SA, Bachireddy P, Schilling B, Galonska C, Zhan Q, Bango C, et al. Cancer-Germline Antigen Expression Discriminates Clinical Outcome to CTLA-4 Blockade. *Cell* (2018) 173(3):624–33. doi: 10.1016/j.cell.2018.03.026
204. Garg AD, Dudek AM, Ferreira GB, Verfaillie T, Vandenabeele P, Krysko DV, et al. ROS-induced autophagy in cancer cells assists in evasion from determinants of immunogenic cell death. *Autophagy* (2013) 9(9):1292–307. doi: 10.4161/auto.25399
205. Buchser WJ, Laskow TC, Pavlik PJ, Lin HM, Lotze MT. Cell-mediated autophagy promotes cancer cell survival. *Cancer Res* (2012) 72(12):2970–9. doi: 10.1158/0008-5472.CAN-11-3396
206. Sun K, Xu L, Jing Y, Han Z, Chen X, Cai C, et al. Autophagy-deficient Kupffer cells promote tumorigenesis by enhancing mtROS-NF-kappaB-IL1alpha/beta-dependent inflammation and fibrosis during the preneoplastic stage of hepatocarcinogenesis. *Cancer Lett* (2017) 388:198–207. doi: 10.1016/j.canlet.2016.12.004
207. Liang X, De Vera ME, Buchser WJ, Romo de Vivar Chavez A, Loughran P, Beer Stolz D, et al. Inhibiting systemic autophagy during interleukin 2 immunotherapy promotes long-term tumor regression. *Cancer Res* (2012) 72 (11):2791–801. doi: 10.1158/0008-5472.CAN-12-0320
208. Lotze MT, Buchser WJ, Liang X. Blocking the interleukin 2 (IL2)-induced systemic autophagic syndrome promotes profound antitumor effects and limits toxicity. *Autophagy* (2012) 8(8):1264–6. doi: 10.4161/auto.20752
209. Li J, Yang D, Wang W, Piao S, Zhou J, Saiyin W, et al. Inhibition of autophagy by 3-MA enhances IL-24-induced apoptosis in human oral squamous cell carcinoma cells. *J Exp Clin Cancer Res* (2015) 34:97. doi: 10.1186/s13046-015-0211-0
210. Lin SY, Hsieh SY, Fan YT, Wei WC, Hsiao PW, Tsai DH, et al. Necroptosis promotes autophagy-dependent upregulation of DAMP and results in immunosurveillance. *Autophagy* (2018) 14(5):778–95. doi: 10.1080/15548627.2017.1386359
211. Noman MZ, Janji B, Kaminska B, Van Moer K, Pierson S, Przanowski P, et al. Blocking hypoxia-induced autophagy in tumors restores cytotoxic T-cell activity and promotes regression. *Cancer Res* (2011) 71(18):5976–86. doi: 10.1158/0008-5472.CAN-11-1094
212. Baginska J, Viry E, Berchem G, Poli A, Noman MZ, van Moer K, et al. Granzyme B degradation by autophagy decreases tumor cell susceptibility to natural killer-mediated lysis under hypoxia. *Proc Natl Acad Sci U S A* (2013) 110(43):17450–5. doi: 10.1073/pnas.1304790110
213. Yamamoto K, Venida A, Perera RM, Kimmelman AC. Selective autophagy of MHC-I promotes immune evasion of pancreatic cancer. *Autophagy* (2020) 2020:1–2. doi: 10.1080/15548627.2020.1769973
214. Meng Q, Zhang Y, Hu LG. Targeting Autophagy Facilitates T Lymphocyte Migration by Inducing the Expression of CXCL10 in Gastric Cancer Cell Lines. *Front Oncol* (2020) 10:886. doi: 10.3389/fonc.2020.00886
215. Noman MZ, Parpal S, Van Moer K, Xiao M, Yu Y, Viklund J, et al. Inhibition of Vps34 reprograms cold into hot inflamed tumors and improves anti-PD-1/PD-L1 immunotherapy. *Sci Adv* (2020) 6(18):eaax7881. doi: 10.1126/sciadv.aax7881
216. Mgrditchian T, Arakelian T, Paggetti J, Noman MZ, Viry E, Moussay E, et al. Targeting autophagy inhibits melanoma growth by enhancing NK cells infiltration in a CCL5-dependent manner. *Proc Natl Acad Sci U.S.A.* (2017) 114(44):E9271–E9. doi: 10.1073/pnas.1703921114
217. Kumai T, Matsuda Y, Ohkuri T, Oikawa K, Ishibashi K, Aoki N, et al. c-Met is a novel tumor associated antigen for T-cell based immunotherapy against NK/T cell lymphoma. *Oncoimmunology* (2015) 4(2):e976077. doi: 10.4161/2162402X.2014.976077
218. Li Y, Wang LX, Yang G, Hao F, Urba WJ, Hu HM. Efficient cross-presentation depends on autophagy in tumor cells. *Cancer Res* (2008) 68 (17):6889–95. doi: 10.1158/0008-5472.CAN-08-0161

219. Li W, Zhou M, Ren H, Hu HM, Lu L, Cao M, et al. Tumor-derived autophagosomes (DRibbles) induce B cell activation in a TLR2-MyD88 dependent manner. *PLoS One* (2013) 8(1):e53564. doi: 10.1371/journal.pone.0053564
220. Twitty CG, Jensen SM, Hu HM, Fox BA. Tumor-derived autophagosome vaccine: induction of cross-protective immune responses against short-lived proteins through a p62-dependent mechanism. *Clin Cancer Res* (2011) 17 (20):6467–81. doi: 10.1158/1078-0432.CCR-11-0812
221. Su H, Luo Q, Xie H, Huang X, Ni Y, Mou Y, et al. Therapeutic antitumor efficacy of tumor-derived autophagosome (DRibble) vaccine on head and neck cancer. *Int J Nanomed* (2015) 10:1921–30. doi: 10.2147/IJN.S74204
222. Gabai VL, Shifrin VI. Feasibility analysis of p62 (SQSTM1)-encoding DNA vaccine as a novel cancer immunotherapy. *Int Rev Immunol* (2014) 33 (5):375–82. doi: 10.3109/08830185.2014.954699
223. Rao S, Yang H, Penninger JM, Kroemer G. Autophagy in non-small cell lung carcinogenesis: A positive regulator of antitumor immunosurveillance. *Autophagy* (2014) 10(3):529–31. doi: 10.4161/auto.27643
224. Lin H, Yan J, Wang Z, Hua F, Yu J, Sun W, et al. Loss of immunity-supported senescence enhances susceptibility to hepatocellular carcinogenesis and progression in Toll-like receptor 2-deficient mice. *Hepatology* (2013) 57 (1):171–82. doi: 10.1002/hep.25991
225. Ramakrishnan R, Huang C, Cho HI, Lloyd M, Johnson J, Ren X, et al. Autophagy induced by conventional chemotherapy mediates tumor cell sensitivity to immunotherapy. *Cancer Res* (2012) 72(21):5483–93. doi: 10.1158/0008-5472.CAN-12-2236
226. Kim S, Ramakrishnan R, Lavilla-Alonso S, Chinnaiyan P, Rao N, Fowler E, et al. Radiation-induced autophagy potentiates immunotherapy of cancer via up-regulation of mannose 6-phosphate receptor on tumor cells in mice. *Cancer Immunol Immunother* (2014) 63(10):1009–21. doi: 10.1007/s00262-014-1573-4
227. Hahn T, Akporiaye ET. alpha-TEA as a stimulator of tumor autophagy and enhancer of antigen cross-presentation. *Autophagy* (2013) 9(3):429–31. doi: 10.4161/auto.22969
228. Li Y, Hahn T, Garrison K, Cui ZH, Thorburn A, Thorburn J, et al. The vitamin E analogue alpha-TEA stimulates tumor autophagy and enhances antigen cross-presentation. *Cancer Res* (2012) 72(14):3535–45. doi: 10.1158/0008-5472.CAN-11-3103
229. Wen Y, Graybill WS, Previs RA, Hu W, Ivan C, Mangala LS, et al. Immunotherapy targeting folate receptor induces cell death associated with autophagy in ovarian cancer. *Clin Cancer Res* (2015) 21(2):448–59. doi: 10.1158/1078-0432.CCR-14-1578
230. Elimam H, El-Say KM, Cybulsky AV, Khalil H. Regulation of Autophagy Progress via Lysosomal Depletion by Fluvastatin Nanoparticle Treatment in Breast Cancer Cells. *ACS Omega* (2020) 5(25):15476–86. doi: 10.1021/acsomega.0c01618

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Bustos, Antunes, Rangel and Chammas. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Oncology

REVIEW
published: 01 December 2020
doi: 10.3389/fonc.2020.603661

The Role of Autophagy in Tumor Immunology—Complex Mechanisms That May Be Explored Therapeutically

Alana Serrano Campelo de Souza[1,2], *Letícia Boslooper Gonçalves*[1,2], *Ana Paula Lepique*[3] *and Patrícia Savio de Araujo-Souza*[1*]

[1] *Laboratório de Imunogenética e Histocompatibilidade (LIGH), Departamento de Genética, Setor de Ciências Biológicas, Universidade Federal do Paraná (UFPR), Curitiba, Brazil,* [2] *Programa de Pós-graduação em Genética, Departamento de Genética, Universidade Federal do Paraná (UFPR), Curitiba, Brazil,* [3] *Laboratório de Imunomodulação, Departamento de Imunologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brazil*

OPEN ACCESS

Edited by:
Daniel Hector Grasso, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

Reviewed by:
Daniela Laura Papademetrio, University of Buenos Aires, Argentina
Eswari Dodagatta-Marri, University of California, San Francisco, United States

***Correspondence:**
Patrícia Savio de Araujo-Souza
psas@ufpr.br
orcid.org/0000-0002-4489-9148

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *07 September 2020*
Accepted: *02 November 2020*
Published: *01 December 2020*

Citation:
de Souza ASC, Gonçalves LB, Lepique AP and de Araujo-Souza PS (2020) The Role of Autophagy in Tumor Immunology—Complex Mechanisms That May Be Explored Therapeutically. Front. Oncol. 10:603661. doi: 10.3389/fonc.2020.603661

The tumor microenvironment (TME) is complex, and its composition and dynamics determine tumor fate. From tumor cells themselves, with their capacity for unlimited replication, migration, and invasion, to fibroblasts, endothelial cells, and immune cells, which can have pro and/or anti-tumor potential, interaction among these elements determines tumor progression. The understanding of molecular pathways involved in immune escape has permitted the development of cancer immunotherapies. Targeting molecules or biological processes that inhibit antitumor immune responses has allowed a significant improvement in cancer patient's prognosis. Autophagy is a cellular process required to eliminate dysfunctional proteins and organelles, maintaining cellular homeostasis. Usually a process associated with protection against cancer, autophagy associated to cancer cells has been reported in response to hypoxia, nutrient deficiency, and oxidative stress, conditions frequently observed in the TME. Recent studies have shown a paradoxical association between autophagy and tumor immune responses. Tumor cell autophagy increases the expression of inhibitory molecules, such as PD-1 and CTLA-4, which block antitumor cytotoxic responses. Moreover, it can also directly affect antitumor immune responses by, for example, degrading NK cell-derived granzyme B and protecting tumor cells. Interestingly, the activation of autophagy on dendritic cells has the opposite effects, enhancing antigen presentation, triggering $CD8^+$ T cells cytotoxic activity, and reducing tumor growth. Therefore, this review will focus on the most recent aspects of autophagy and tumor immune environment. We describe the dual role of autophagy in modulating tumor immune responses and discuss some aspects that must be considered to improve cancer treatment.

Keywords: macroautophagy, tumor microenvironment, antitumor immunity, tumor immune evasion, onco immunology

INTRODUCTION

According to the Cancer Immune Edition hypothesis, tumor and immune cell interactions go through three phases: elimination, equilibrium, and evasion. During cancer development, the immune system recognizes molecular changes in transformed cells and eliminates most or all of them, avoiding tumor progression. Genetic alterations that cause cell transformation generate neoantigens for immune recognition, leading to T lymphocyte activation, which can prevent tumor outgrowth, through cytotoxic activity and interferon-gamma (IFN-γ) signaling (1, 2). At the same time, less immunogenic mutations or mutations that lead to loss of the antigen recognized by the immune system allow tumor cells to escape from elimination mechanisms. As genetic alterations accumulate, generating oncogenes and preventing the expression of tumor-suppressor genes, transformed cells gain proliferative advantages, and again escape immunosurveillance, leading to tumor progression (3, 4).

The interplay between tumor and other cells composing the tumor microenvironment (TME) is determinant for tumor growth, maintenance, metastasis, and response to therapy. TME is composed of stromal cells (fibroblasts, pericytes, mesenchymal and endothelial cells), extracellular matrix (ECM), and immune cells, such as natural killer (NK) cells, tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and T and B lymphocytes. During cancer progression, tumor cells display genetic and phenotypic diversity, changing cellular metabolism and, consequently, the TME (5, 6).

Generally, TME displays low levels of oxygen and nutrients, and high production of reactive oxygen species (ROS), crucial factors for autophagy activation. Autophagy is a natural cellular survival process, usually activated to maintain cellular homeostasis (7, 8). Despite that, recent studies have suggested that autophagy is also important for cancer development and progression, neurodegenerative and infectious diseases, once it can affect immune cells and modulate immune responses (9–11).

In this review, we will present the major mechanisms by which the immune system interferes in the TME, and how autophagy can influence it. Then we will focus on the modifications of cancer immune responses in TME influenced by autophagy and how it can affect cancer therapy.

CANCER IMMUNE RESPONSE AND TUMOR MICROENVIRONMENT

Besides the TME elements already mentioned, soluble molecules as cytokines, metabolites, and inflammation mediators also contribute to the interaction among the cellular elements. These biochemical signals orchestrate cell death, proliferation, survival, and other cells recruitment. Leukocyte activity is essential in cancer progression (reviewed in 12). Since Rudolph Virchow has described the presence of lymphoreticular infiltrate in human tumors, it was discovered that leukocytes play an essential role in tumor progression, either by eliminating tumor cells or by facilitating progression and growth (13).

Immune responses can inhibit tumor growth and even eliminate tumor cells completely. However, chronic inflammation is considered a risk factor for many types of cancers (reviewed in 12). An interesting example of this dual role is HIV infection, AIDS (Acquired Immune Deficiency Syndrome), and cancer risk. In the early '90s, patients with AIDS were at high risk of Kaposi sarcoma and non-Hodgkin lymphoma development, in part due to cellular and molecular mechanisms, but in large part due to immunodeficiency. Highly active antiretroviral therapy (HAART) decreased AIDS-related cancer (14) by increasing T lymphocyte levels and consequently immune responses. Still, HAART treated HIV infected patients display chronic inflammation and early aging, with increased plasma levels of interleukin-(IL)6 and C reactive protein, which have a role in carcinogenesis. Indeed, HAART treated patients display increased risk to develop AIDS-unrelated cancer, such as cervical, lung, anal cancer, and Hodgkin lymphoma (14, 15).

The immune responses mediated by NK cells, $CD8^+$ T lymphocytes, and $CD4^+$ T helper (Th) 1 and Th17 lymphocytes are considered cytotoxic responses. These cells can control tumor growth through either directly killing tumor cells, as NK and $CD8^+$ T cells, or indirectly, in the case of $CD4^+$ T cells, which secrete cytokines capable of activating other effector leukocytes (16–18).

NK cells and $CD8^+$ T lymphocytes are *bona fide* cytotoxic cells. NK cells are innate lymphoid cells that recognize target cells through activating and inhibitory receptors. The signaling triggered by these sets of receptors determines the cytotoxic activity. Among the inhibitory receptors, there are the killer immunoglobulin-like inhibitory receptors (KIRs), which recognize human leukocyte antigen (HLA) class I molecules and

Abbreviations: IFN-γ; interferon gamma; TME, tumor microenvironment; ECM, extracellular matrix; NK, natural killer; TAMs, tumor-associated macrophages; MDSC, myeloid-derived suppressor cell; ROS, reactive oxygen species; AIDS, Acquired Immune Deficiency Syndrome; HAART, highly active antiretroviral therapy; IL, interleukin; Th, T helper; KIR, killer immunoglobulin-like inhibitory receptor; HLA, human leucocyte antigen; TCR, T cell receptor; DC, dendritic cell; Treg, regulatory T cells; IRF1, interferon regulatory factor 1; HDAC, histone deacetylase; IFN-I, type I interferon; TNF-α, tumor necrosis factor alpha; CTLA-4, cytotoxic T lymphocyte associated protein 4; IDO, indoleamine-2,3-deoxygenase; PD-L1, Programmed cell death-ligand 1; APC, antigen presenting cell; PD-1, programmed cell death-1; ITIM, immunoreceptor tyrosine-based inhibitory; PD-L2, programmed cell death ligand-2; CMA, chaperone-mediated autophagy; LC3, light chain protein-3; Hsc 70, heat shock cognate 70; LAMP, lysosomal-associated membrane protein; HIF, hypoxia-inducible factor; HREs, hypoxia response elements; BNIP3, BCL2 interacting protein 3; BNIP3L, BCL2 interacting protein 3 like; Bcl-2, B-cell lymphoma 2; ATG, autophagy-related genes; AMPK, adenosine monophosphate-activated protein kinase; mTOR, mammalian target of rapamycin; TAA, tumor-associated antigens; MHC, major histocompatibility complex; TILs, tumor infiltrate lymphocytes; α-TEA, alpha-tocopheryloxyacetic acid; LLC, Lewis lung carcinoma; TLR, toll-like receptor; PDAC, pancreatic ductal adenocarcinoma; TIM-4, T cell immunoglobulin and mucin domain protein-4; CQ, chloroquine; TNBC, triple negative breast cancer; LDHA, lactate dehydrogenase A; G-CSF, granulocyte colony- stimulating factor; LAP, LC3-associated phagocytosis; NSCLC, non-small cell lung cancer; GB, glioblastoma; PC, pericytes; NBD, nitrobenzoxadiazole; MTX, mitoxantrone; ICD, immunogenic cell death; DAMPs, damage-associated molecular patterns; CRT, calreticulin; ATP, adenosine triphosphate; CT, chemotherapy; HCQ, Hydroxychloroquine sulfate; MAGE, melanoma-associated antigen; HNSCC, head and neck squamous cell carcinoma.

CD94/NKG2A, which specifically binds to the non-classical HLA-E molecule. The last one causes NK inhibition to ensure that normal cells cannot be lysed. However, transformed cells that downregulate the HLA-I surface molecules are not able to inhibit NK cells. The stimulatory receptors bind to stress-inducible molecules in the target cell surface, as sialic acid, Fcγ, adhesion molecules, and others, to trigger cytotoxic activity. Displaying a different strategy for target recognition, $CD8^+$ T lymphocytes activation depends on TCR (T cell receptor) binding to specific antigens presented by classical HLA-I molecules in target cells (19). In spite of the different development and recognition receptors, both NK and $CD8^+$ T cells display similar cytotoxic mechanisms, leading to the activation of cell death pathways in cancer cells (20).

Th1 and Th17 cells can either assist in $CD8^+$ T lymphocytes and dendritic cells (DCs) activation, through CD40L signaling, and cytokine secretion as IL-2, or activate other effector cells, such as macrophages, neutrophils, NK cells through IFN-γ and tumor necrosis factor-alpha (TNF-α). Moreover, TNF-α, through its receptor, can trigger cell death, and IFN-γ and cytokines secreted by Th17 cells, through activation of stromal cells, can stimulate ROS production and neutrophils, enhancing the cytotoxic effects on cancer cells (21).

These antitumor responses are counteracted by tolerogenic responses, enabling tumor growth. There are several known immune escape mechanisms. Chemokines secreted by cells in the TME favors the recruitment of MDSCs and regulatory T cells (Treg), well-characterized suppressors of effector T lymphocytes function. Moreover, it is well known that cancer cells display reduction in antigen presentation potential, decreasing tumor cell recognition by CD8 T lymphocytes. One classic example, from a virus associated cancer is the HPV E7 oncoprotein, which binds to interferon regulatory factor 1 (IRF1) in the IFN type I (IFN-I) signaling pathway, and recruits histone deacetylase (HDAC) to the promoter sequences responsive to IRF1, repressing genes that otherwise would be transcribed in response to the virus (22). IFN-I are important activators of innate responses, as well as antigen-presenting activity, therefore playing a role in T lymphocyte activation and phenotype (23). More recently, it has become clear that human oncogenes also play a role in immune escape mechanisms (24). Stabilization of β-catenin, in the Wnt pathway, for example, reduces the expression of CCL4, a chemokine that attracts DCs, impairing tumor antigen presentation (25).

Oncogenes also drive the reprogramming of tumor cell metabolism, the so-called Warburg effect. Tumor cells display different metabolic strategies to maintain energy production and catabolism at a rate to allow continuous cell proliferation. Some cells use glycolysis almost exclusively, while others also required amino acids and fatty acids as well, and keep the Krebs cycle and oxidative phosphorylation active. In either case, tumor cells usually increase the glucose uptake and secrete lactate in higher concentrations than other cells in the body (26). Both the decrease in glucose and the increase in lactate concentration have consequences for immune responses. Activated T lymphocytes and M1 macrophages display a metabolic profile similar to tumor cells, therefore, dependent on glucose. Low glucose concentration inhibits T lymphocyte proliferation and macrophage function. Additionally, lactate is a regulatory molecule, modulating the phenotype of DCs, inducing suppressor phenotype on macrophages, and inhibiting T lymphocytes (27).

Besides tumor cell-intrinsic metabolism, other cells in the TME also display metabolic pathways that lead to tolerance. DCs, the essential population for naive T lymphocyte activation, can acquire tolerogenic phenotype due to signals from tumor cells, but also from binding, *via* CD80 or CD86, to cytotoxic T lymphocyte-associated protein 4 (CTLA-4) expressed by Treg. The signal triggered by this interaction promotes indoleamine-2,3-deoxygenase (IDO) expression in DCs. This enzyme, which physiological function is the protection of immune-privileged tissues, catabolizes the reaction that converts the essential amino acid tryptophan in kynurenine, which through the aryl hydrocarbon receptor, promotes regulatory phenotype in T lymphocytes (28). Therefore, this mechanism works as an amplification of the regulatory cycle in the TME. Furthermore, tumor cells can also overexpress IDO, as observed in oral squamous cell carcinoma from smoker patients (29). In general, IDO expression depends on IFN-γ stimulation, which in cancer, characterizes it as a negative feedback mechanism for effector immune responses.

Programmed cell death-ligand 1 (PD-L1) is also an inhibitory molecule expressed upon IFN-γ stimulation, both in antigen-presenting cells (APCs) and cancer cells (30). The receptor for PD-L1, programmed cell death-1 (PD-1), is upregulated upon T lymphocytes activation. Several transcription factors, such as NFAT, AP-1, FoxO1, and NFκB, mediate the *PDCD1* expression. Moreover, chromatin changes are also important to control PD-1 expression and are observed in exhausted $CD8^+$ T cells (31). PD-1 contains immunoreceptor tyrosine-based inhibitory (ITIM) domains and can inhibit TCR signaling, rendering T cells inactive. PD-1 expressing stem-like $CD8^+$ memory T cells can be found in lymphoid follicles in the tumor (32). These cells, when activated, differentiate in exhausted cells. There are two PD-1 ligands: CD274 (PD-L1), which has a basal expression in several cell types, and programmed cell death ligand-2 (PD-L2), which expression is usually limited to DCs and macrophages. A variety of cancers display constitutive PD-L1 expression, which can be triggered by genetic and epigenetic alterations in its promoter region, cytokine stimulation, such as IFN-γ and IL-6, growth factors, hypoxia, among others (28). The PD-1/PD-L1 signaling, which induces T cell exhaustion, is an important effect resulting from the chronicity of antigen presentation in cancer. Whenever antigens are chronically presented, negative feedback mechanisms are activated to protect the organism. This protective response is usurped by cancer to create an immune-privileged situation, and immune evasion (33).

As mentioned before, CTLA-4 binds to the co-stimulatory molecules CD80 and CD86. It competes with the activating receptor CD28, which also binds to these molecules, but with lower affinity. CD28 signaling is essential for T lymphocyte activation since it triggers the PI3K/Akt pathway and causes

stabilization of the antigen activation signal. Not only CTLA-4 competes for biding to co-stimulatory molecules, but also, through a process called trans-endocytosis, this biding removes CD80 and CD86 from the APC surface, eliminating the possibility of CD28 activation, and consequently preventing T cell activity (34).

Many of these mechanisms can happen simultaneously in the TME, in a dynamic process that varies through time. To add to this complex situation, other factors should also be considered. Some tumors are very immunogenic, and tumor antigen-specific T lymphocytes can be found in the TME, where it can also be observed evasion mechanisms and chronic antigen signaling that can, eventually, inhibit anti-tumor responses. When immune responses persist, it results in chronic inflammation leading to cancer progression. Other tumors are less immunogenic and recruit mainly myeloid cells, which display a tolerogenic phenotype, helping cancer cells meet their metabolic demands, and promoting angiogenesis. M2 macrophages, for example, display arginase activity, causing conversion of arginine to ornithine, which is a substrate to the synthesis of polyamines, necessary for catabolism and cell proliferation (35).

AUTOPHAGY MODULATES TUMOR IMMUNE ENVIRONMENT

Autophagy

Autophagy is a survival cellular process in which organelles and other cytoplasmic components are directed to the lysosomes for degradation (7, 36). This mechanism is highly conserved in eukaryotic cells and its activation occurs in face of starvation, hypoxia, and/or oxidative stress conditions (8). Up to now, three classes of autophagy are known: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). In macroautophagy, an isolation membrane enclosures a portion of the cytoplasm, molecules, and organelles, forming a double-membrane vesicle associated with light chain protein-3 (LC3), called an autophagosome. LC3 is processed and cleaved, generating LC3-I, which receives carboxyl glycine radical and turns into LC3-II. LC3-II acts as a receptor in autophagosome membrane binding to p62, through the LC3-interacting region. P62 is a multidomain protein, involved in the cell death and survival process, which delivers ubiquitin radicals to LC3-II. This induces autophagosome-lysosome fusion to form an autolysosome, autophagy is then activated and p62 is degraded (37, 38). In microautophagy, an invagination of the lysosome membrane engulfs cytoplasmic compounds, in a similar process to endosome formation, producing a multivesicular body (39). Conversely, CMA is a type of autophagy used to degrade specific soluble proteins. A cytosolic substrate is recognized by the chaperone protein heat shock cognate 70 (Hsc 70), which binds to lysosomal-associated membrane protein-(LAMP) 2A in the lysosome membrane to transport this substrate into the lysosome lumen (40).

Macroautophagy is the main type of autophagy, therefore it will be referred to just as autophagy. In response to hypoxia and oxidative stress, hypoxia-inducible factors (HIF) 1 and 2 bind to hypoxia response elements (HREs), leading to the transcription of several genes that are involved in angiogenesis, metastasis, cell survival, immune escape, and autophagy pathways. Activation of HIF-1 subunit-1 leads to an increase in BCL2 interacting protein 3 (BNIP3) and BCL2 interacting protein 3 like (BNIP3L) expression levels. These factors are responsible for breaking the connection between Beclin1 (BECN1) and B-cell lymphoma 2 (Bcl-2), an inhibitory complex that prevents autophagy (41). Another way to induce autophagy in a hostile cellular environment is through the activation of autophagy-related genes (ATG) (42) and 5'-adenosine monophosphate-activated protein kinase (AMPK) (43). AMPK is a nutrient availability sensor and can regulate oxidative and glycolytic metabolism. It can also activate the autophagic recycling of cellular components to balance cellular energy supply. In autophagy activation, ATG and AMPK, independently of BNIP3 and BNIP3L, downregulate the mammalian target of rapamycin (mTOR), which drives autophagosome formation (43).

Despite being a natural process to maintain cellular homeostasis, autophagy activity has been described to contribute to the progression of many human diseases, such as some neurodegenerative disorders, infectious diseases, and cancer. In 1999, it was described that mono-allelic deletions and decrease in expression of *Beclin1*, on MCF7 human breast carcinoma cells, contributed to tumorigenesis in nude mice, indicating that autophagy could inhibit tumor growth (9). It is reasonable to assume that autophagy could stop the transformation process by eliminating oncogenic, aggregated, or erroneously folded proteins (44). Nevertheless, tumor cells and components of TME can induce autophagy to survive hostile conditions and suppress immune responses, helping tumor growth and proliferation (reviewed in 45). Thus, its role in cancer development remains unclear, and the aspects of how autophagy can modulate immune components of TME will be reviewed in the next topics.

Autophagy and Antitumor Immune Response

The Dual Role of Autophagy in Antigen Presentation

Studies have shown the influence of autophagy in antigen presentation (46–49), as well as in the anti-tumor adaptive immune response activation (50). As shown in **Figure 1**, adaptive anti-cancer immune responses are triggered by endogenous tumor-associated antigens (TAA) presented to T lymphocytes *via* the major histocompatibility complex (MHC) context by DCs (reviewed in (51, 53). Normally, MHC-I presents intracellular antigens, such as the ones derived from self-proteins and viral proteins, to $CD8^+$ T lymphocytes while extracellular antigens are generally presented to $CD4^+$ T lymphocytes by MHC-II (reviewed in 54). Effective tumor antigen presentation and the consequent effector T lymphocyte responses and access to the TME are essential for clinical responses to immunotherapy (55) and are associated with positive clinical outcomes (56). Reduction in MHC expression and expression of non-classical molecules is frequently observed in different types of cancers, leading to compromised antigen presentation and/or immune

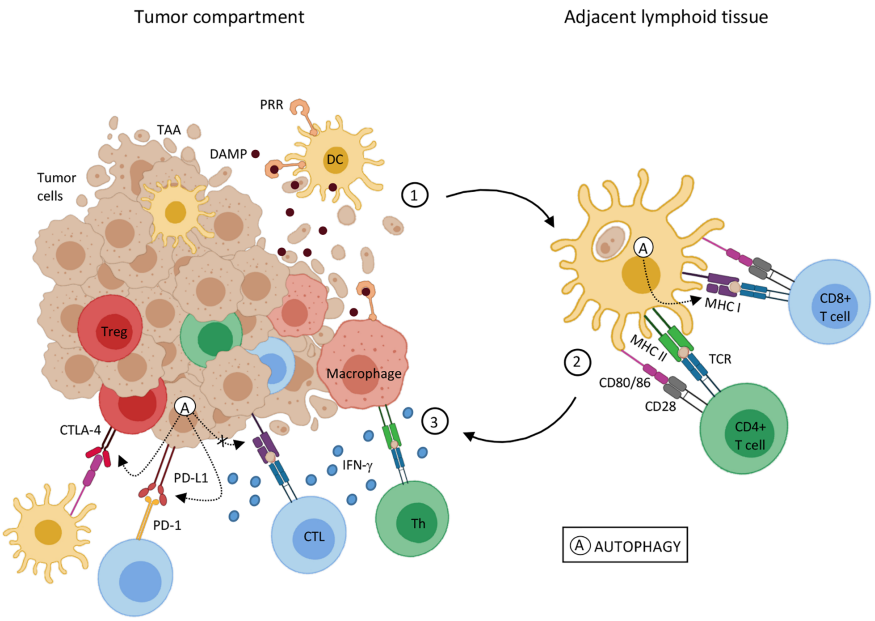


FIGURE 1 | Autophagy influence on tumor-associated antigen presentation 1. Genetic alterations that cause cell transformation can also generate tumor-associated antigens (TAA) for immune recognition. Tumor cell death is an antigen source for antigen-presenting cells, such as dendritic cells (DC) and macrophages. DC activation by DAMPs and antigen processing leads to an upregulation of costimulatory molecules and MHC on DC surface, the cardinal signals for T lymphocyte activation, and migration to adjacent lymphoid tissue. 2. Mature DCs present TAA through MHC-I and MHC-II to naïve CD8+ T cells and CD4+ T cells, respectively. Antigen recognition results in T lymphocyte differentiation in effector cells (CTL e Th) and migration to the tumor site. 3. In the tumor microenvironment, upon TAA recognition through TCR interaction with MHC-I and MHC II, CTL and Th lymphocytes, respectively, trigger cytotoxic mechanisms, as interferon-gamma (IFN-γ) mediated ones. Despite that, inhibitory molecules, such as PD-1 and CTLA-4 in T cells, and PD-L1 in TME can interfere in T cell activation and function. IFN-γ stimulation can result in PD-L1 expression in both APCs and tumor cells, inhibiting T cell function. CTLA-4 expressed by regulatory T lymphocytes (Treg), through binding to co-stimulatory molecules, CD80/86, induces tolerogenic phenotype on DCs, amplifying the regulatory mechanisms in the TME. Autophagy (A) can either help or disturb the antigen presentation and T cell activation pathway. Autophagic activity on DCs seems to increase MHC-I expression, thus enhancing antigen presentation. On the other hand, autophagy activation on tumor cells may promote a reduction in MHC-I and an increase in PD-1 and CTLA-4 expression, leading to tumor progression. Sources (11, 28, 34, 47, 49, 51–56).

evasion (reviewed in 52), which can influence tumor progression and resistance to immunotherapy (57).

Effective Antitumor Immune Responses

Effective antitumor responses are dependent on potent antigen presentation and leukocyte infiltrates enriched with effector $CD8^+$ T cells (49). Autophagy activation in DCs may improve antigen presentation and stimulate cytotoxic responses mediated by $CD8^+$ T lymphocytes (47, 49). For example, nano-activators conjugated to antigens were used to stimulate DCs, triggering anti-tumor T cell responses in mice. It has been shown that nano-activators treated DCs, through autophagy-dependent mechanisms, could increase antigen presentation and cross-presentation to T lymphocytes, increasing effector $CD8^+$ T tumor infiltrating lymphocytes (TILs) (49). Additionally, experimental data has shown that semi-synthetic vitamin E derivative alpha-tocopheryloxyacetic acid (α-TEA) could modulate autophagy in tumor cells from both Lewis lung carcinoma (LLC) and murine mammary tumor, improving antigen cross-presentation by DCs and triggering tumor antigen-specific $CD8^+$ T lymphocytes responses. Treatment with α-TEA resulted in LC3-II increase, both *in vitro* and *in vivo*, indicating autophagic activity. These authors also found that α-TEA-generated autophagosome-enriched fraction (α-TAGS) was a competent tumor antigen carrier, which stimulated antigen cross-presentation mediated by DCs to $CD8^+$ T cells and stimulated $CD8^+$ T cell proliferation in an autophagy-dependent fashion. Overall, these findings demonstrated a new mechanism of immune activation by α-TEA, which stimulated tumor cell autophagy and antigen cross-presentation to $CD8^+$ T cells (47).

Autophagy can also create new epitopes arising from stress-induced post-translational modifications, which could increase immune recognition (46). It has been shown that citrullination – the conversion of arginine residues to citrulline - can take place in cells during autophagy induced by stress and, in inflammatory conditions, it can result in MHC-II presentation of citrullinated epitopes to $CD4^+$ T cells (46). Although autophagy modulation has not been directly investigated, the combination of citrullinated peptide based vaccine with TLR ligand adjuvant promoted a Th1 anti-tumor response in melanoma and ovarian cancer mouse models. $CD4^+$ T TILs were associated with tumor regression. Interestingly, they also observed a Th1 response to the citrullinated peptides in ovarian cancer patients (58). Collectively, these findings indicated that autophagy is associated with efficient antigen presentation in different types

of cancer. As it increases antigen availability and enhances T cell activation, it favors cytotoxic responses and clearly can act to inhibit cancer progression.

Immunosuppression: Autophagy Disrupts Antigen Presentation

Autophagy may also play a negative role in antigen presentation, facilitating tumor evasion from $CD8^+$ T cells in both pancreatic ductal adenocarcinoma (PDAC) and melanoma (11, 59). PDAC displays low levels of MHC-I surface molecules. In these tumor cells, knockdown of the autophagy cargo receptor gene, *NBR1*, increased MHC-I surface expression, confirming the implication of NBR1-mediated autophagy-lysosomal pathway in the process. The authors showed that NBR1 targeted MHC-I for lysosomal degradation. Mouse PDAC cells expressing an autophagy inhibitor, restored MHC-I membrane expression, improved antigen presentation, and $CD8^+$ TILs, leading to a reduction in tumor growth. These findings indicated that high levels of MHC-I at PDAC cell surface after autophagy inhibition were required to increase $CD8^+$ T cell infiltration and to kill the tumor cells (11).

The activation of autophagy pathways has also been described in macrophages and DCs infiltrating B16F10 mouse melanoma. These tumors normally express T cell immunoglobulin and mucin domain protein-4 (TIM-4). Autophagy initiates when TIM-4 binds to AMPK-α1. This activation promotes the degradation of TAA through the lysosomal pathway, which led to a decrease in antigen presentation and, consequently, in specific anti-tumor $CD8^+$ T cells. TIM-4 blockade with a monoclonal specific antibody resulted in autophagy inhibition and improvement in antigen cross-presentation and IFN-γ production (59). Moreover, chloroquine (CQ), a known autophagy inhibitor, combined with low concentrations of 5-fluorouracil (5-FU), increased DCs maturation and activation in HCT-116 colorectal cancer cells, enhancing $CD8^+$ T lymphocyte stimulation (60). Together, these results suggested that autophagy can impair antigen presentation by interfering with different key steps in this process.

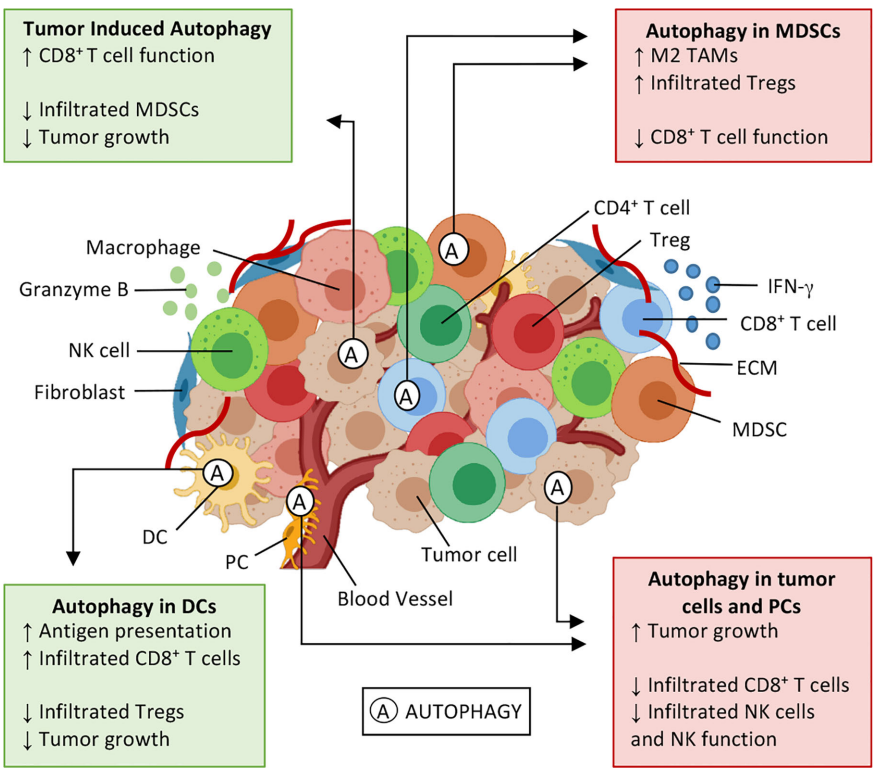


FIGURE 2 | Effects of autophagy on the tumor microenvironment. Tumor microenvironment (TME) is composed by cytokines (e.g. IFN-γ), extracellular matrix (ECM), and several cell types: tumor cells, fibroblasts, and immune cells, such as natural killer (NK) cells, $CD8^+$ T and T helper (Th) lymphocytes, T regulatory (Treg) cells, myeloid-derived suppressor cells (MDSC), dendritic cells (DCs), macrophages, and pericytes (PC). Autophagy (A) is a cellular survival mechanism, which is activated in stressful conditions, can be activated in TME. Autophagy can either enhance antitumor immune responses (green boxes) or induce an immunosuppressive environment (red boxes), thus playing a dual role in cancer development and progression. Autophagy activation in DCs enhances antigen presentation and results in an enrichment of $CD8^+$ T tumor-infiltrating lymphocytes, also decreasing infiltrated Treg and tumor cell proliferation. Similar outcomes can be seen when IFN-γ induces autophagy on tumor cells, and elevated extracellular potassium induces autophagy on $CD8^+$ T cells. In these situations, it is possible to observe a decrease in infiltrated MDSCs and T lymphocytes expressing PD-1, thus inhibiting tumor growth. Contrarily, the autophagy activation in myeloid cells and tumor cells has the opposite effect, favoring an immunosuppressive profile of TME, leading to tumor cell proliferation, through M2 macrophage polarization, enhancing Treg infiltration and inhibitory molecules (PD-1 and CTLA-4) expression. Sources (5, 6, 11, 47, 49, 61–67).

TME Induces Autophagy

Not only autophagy modulates TME components, but the opposite is also true. As shown in **Figure 2**, cytokines and metabolic conditions may also promote or inhibit autophagy and influence the tumor immune response. IFN-γ can induce autophagy in gastric cells, thus inhibiting carcinogenesis (61). Gastric cancer is usually associated with chronic inflammation. Transgenic mice that overexpress IFN-γ in the gastric mucosa (H^+/K^+-ATPase-IFN-γ and H^+/K^+-ATPase-IL-1β; IFN-γ) displayed protection against gastric dysplasia in comparison to controls. The authors observed that gastric cells displayed lower proliferation rates and T cell apoptosis dependent on IFN-γ expression. Furthermore, transgenes resulted in increased levels of LC3-II and Beclin-1 mRNA and protein, in the stomach, indicating autophagy activation. Additionally, transgenic animals showed higher apoptotic T cells, concurrently with inhibition of IL-6, IL-1β; and TNF-α production, and presented less chronic inflammation (61).

Besides the cellular components and soluble mediators, TME also display metabolic disorders such as aerobic glycolysis (68), oxygen deprivation, and higher levels of extracellular potassium (62), which are stressful conditions that can also influence cellular autophagy. Activation of autophagy in T lymphocytes exposed to elevated potassium concentrations reduced acetylation of the promoter and enhancer regions of T cell effector- and exhaustion-markers *Ifng* and *Pdcd1*. Potassium exposed T lymphocytes adoptively transferred to B16 melanoma bearing mice resulted in T cells persistence in the TME, driving tumor regression, and improving animal survival (62). Furthermore, metabolic conditions of TME, such as elevated extracellular potassium levels, were responsible for autophagy activation on T-cells and boosted antitumor responses (62).

Despite that, tumor metabolism can play tricks on antitumor immune responses, such as the ones with elevated glycolytic metabolism, that seems to inhibit autophagy pathways and fuel MDSCs development, leading to an immunosuppressive environment (68). MDSCs and Treg cells suppress T lymphocyte effector functions in the TME and are enriched in tumors with elevated glycolytic metabolism, such as triple-negative breast cancer (TNBC). These tumors secrete high levels of G-CSF, which stimulates MDSCs, and lower expression levels of LC3 mRNA. Inhibition of the glycolytic enzyme lactate dehydrogenase A (LDHA) in 4T1 and Py8119 TNBC cells using target-directed short-hairpin(sh), restored tumor autophagy, and reduced MDSC infiltration in tumors growing in BALB/C and C57/BL6 mice, respectively. The mechanism was dependent on AMPK-ULK1 signaling, which was impaired by glycolysis. At the same time, glycolysis and AMPK-ULK1 inhibition increased G-CSF expression. Consequently decreasing IFN-γ^+ and TNF-α^+ effector $CD8^+$ T TILs and in tumor-draining lymph nodes, resulting in an immunosuppressive environment. Consequently, autophagy boosted antitumor response mediated by effector $CD8^+$ T cells (68). These results described above indicate that autophagy may be an important mechanism in tumor immune responses. Sometimes, directly improving the cytotoxic activity of T cells, and others, modulating the immunosuppressive components of TME, such as infiltrated MDSCs and T-cell exhaustion-markers, leading to tumor elimination and better survival rates.

Autophagy and Immune Evasion Mechanisms

Despite the evidence that autophagy can improve antitumor immune responses, it can also inhibit both innate and adaptive responses, leading to cancer immune evasion.

Autophagy in Myeloid Cells Induces an Immunosuppressive TME

It is known that MDSCs and M2 macrophages are components of the immunosuppressive compartment of TME. The autophagic activity in MDSCs has been described by different research groups (63, 64) and has been associated with antigen presentation and cytotoxic T cell function impairment, as well as to M2 macrophage polarization and Treg recruitment (**Figure 2**) (48, 63).

The importance of a non-canonical autophagy pathway, the LC3-associated phagocytosis (LAP), in TME was demonstrated using Cre-lox based ablation of several genes in myeloid cells of immunocompetent mice (C57BL/6). The ablation of *Becn1*, *Vps34*, *Atg5*, *Atg7*, or *Atg16l1* impacted on both conventional autophagy and LAP pathway. While myeloid cells deficient in *Fip200*, *Ulk1*, or *Atg14* lacked only the canonical autophagy pathway, and the absence of *Rubicon* or *Nox2* affected only the LAP pathway (63). In the LLC mouse model, the absence of LAP ($Rubcn^{-/-}$) in myeloid cells increased the co-stimulatory molecule CD86 and reduced CD206, a mannose receptor associated with M2-like phenotype. In this model, the reduction on M2-like TAMs improved $CD8^+$ and $CD4^+$ T cell IFN-γ production, increased IFN-I, and IL-1β, although no quantitative alteration in the frequency of TILs was observed (63), suggesting modulation of T cell activity rather than proliferation or recruitment.

Similarly, an increase in autophagy activity was observed in MDSCs from melanoma patients and melanoma experimental model. Functional autophagy was measured by the expression of LC3, LAMP-1, and SQSTM1/p62, and colocalization analysis with p62 and LC3 in MDSCs isolated from melanoma patients (stages III and IV) and clinically healthy controls' peripheral blood. MDSCs from melanoma patients, and also from mice with melanoma, showed higher levels of functional autophagy. In $LysM^{cre}Atg5^{fl/fl}$ mice, in which myeloid cells lacked *Atg5* expression, there were less Treg TILs, tumor cell proliferation rate reduction, and a significant increase in both MHC-II expression and IFN-γ production (64). Again, an indication that autophagy activity in TME myeloid cells plays a role in these cells' immunosuppressive functions.

Autophagy Disrupts the Cytotoxic Activity of TME

TME is a hostile environment where autophagy can lead to degradation of cytotoxic molecules (granzyme B and IFN-γ),

expression of T cell exhaustion markers, and quantitative changes in TILs.

NK cells release granules containing perforins and granzymes as part of their effector mechanism. In MCF7 breast cancer cells, granzyme B suffered lysosome degradation, after hypoxia-induced autophagy, which inhibited NK cell-mediated tumor cell lysis (65). Likewise, *BECN1* inhibition increased functional NK cell tumor infiltration in a melanoma mouse model. The higher frequency in infiltrating NK cells was correlated with an increase in CCL5 secretion, which is an important chemokine for NK cell proliferation and activation. The enhancement of NK cell function, after inhibition of tumor-autophagy, caused tumor regression and predicted improved patient survival (66), suggesting that tumor cell-autophagy can be a resistance mechanism to NK cell activity.

The impact of autophagy in cytotoxic immune responses also influences $CD8^+$ and $CD4^+$ T cells, although there is no consensus about this topic in the literature. *Atg5* deficient mice displayed a reduction of $CD8^+$ TILs in mammary and colorectal cancer models. Despite that, among the $CD8^+$ T lymphocytes recruited to the tumors, around 80% exhibited memory phenotype and were positive for IFN-γ and TNF-α, leading to tumor rejection (69). Moreover, in the non-small cell lung cancer (NSCLC) mouse model, *Atg5* tissue-specific depletion promoted tumor initiation through a mechanism dependent on Treg recruitment to the TME (48). These findings indicated that autophagy may have a positive effect on cytotoxic immune responses, through the reduction in Treg frequency in the TME and increase in IFN-γ and TNF-α release.

On the other hand, the interaction with human glioblastoma (GB) cells induced *Lamp2a* mRNA and protein expression in mouse brain pericytes (PC). Engraftment of PC from *Lamp2a* knockout mice (PC-KO) into GB grown in mice led to central-memory ($CD44^+$, $CD62^+$) $CD4^+$ and $CD8^+$ T TILs and better $CD4^+/CD8^+$ ratio of 2:1 than in tumors engrafted with wild type (WT) PC. Moreover, only 2% of TILs expressed PD-1 and CTLA-4 and there was a lower GB cell proliferation rate than in GB/PC-WT control mice. Compared to GB/PC-KO, GB/PC-WT mice displayed increased IL-2 levels, tumor proliferation, $FoxP3^+PD\text{-}1^+CTLA\text{-}4^+$ TILs, higher *Tgfβ*, and *Il10* mRNA expression levels, and a $CD4^+/CD8^+$ ratio of 4:1. Collectively, these results demonstrated that CMA activity in PC favored an immunosuppressive environment in response to GB cells (64).

Inhibitory molecules expression is associated with $CD8^+$ T cell exhaustion phenotype, and also resistance to targeted therapy and autophagy inhibitors. $PD\text{-}L1^+$ A375 melanoma cells showed resistance to BRAF inhibitor Vemurafenib and CQ, but not to nitrobenzoxadiazole (NBD). Both CQ and NBD inhibit autophagy, however, NBD acts at multiple levels, targeting not only the late stages of autophagy but also different apoptotic pathways (70). These findings indicate that autophagy may act as an immunosuppressive mechanism, affecting cytotoxic cells—NK and T lymphocytes—functionally and quantitatively. Additionally, autophagy modulates immune checkpoints expression, leading to T cell exhaustion phenotype and resistance to treatment.

AUTOPHAGY AND CANCER THERAPY

Autophagy can be a preventive mechanism to malignant transformation in healthy cells, but in transformed cells this mechanism can contribute to cancer progression. Autophagy's main roles in tumorigenesis and cancer therapy were reviewed elsewhere (71). In this section, we will focus on the interplay between autophagy and immune responses in cancer therapies in different experimental models.

Autophagy and Conventional Therapies

Traditionally, cancer therapy is based on direct toxicity to tumor cells. But the importance of immune responses to the efficacy of traditional therapies can be observed in mouse models, in which T lymphocytes are necessary for tumor growth reduction by chemotherapeutic agents (72). Recently, it has become clear that several chemotherapeutic agents, such as doxorubicin and mitoxantrone (MTX), and even radiotherapy, may induce tumor cell immunogenic cell death (ICD) (72–74). ICD is characterized by the release of damage-associated molecular patterns (DAMPs) and consequent elicitation of immune responses. The pre-apoptotic surface exposure of calreticulin (CRT) is considered the "eat me" (immunogenicity) signal for DCs, influencing antigen presentation to T lymphocytes (73). Likewise, adenosine triphosphate (ATP) secreted or leaked to the extracellular milieu is considered a "find me" signal. The chromatin-binding protein high mobility group B1 (HMGB1) is another DAMP exposed after chemotherapy by post-apoptotic release. It induces TLR4-MyD88 signaling on DCs facilitating antigen processing and presentation (74, 75).

The ATP lysosomal secretion depends on autophagy, as autophagy-deficient CT26 colon carcinoma mouse cells (due to the lack or decrease in *Atg5* or *Atg7* expression) released lower amounts of ATP in response to MTX chemotherapy. The lower ATP release resulted in DCs recruitment impairment and consequent lack of T cell priming to elicit an anti-tumor immune response (76). Extracellular ATP binds to surface purinergic receptors (such as P2YR2 receptors) on immature DC precursors (77), promoting DC maturation and recruitment to the tumor site in lung cancer mouse model. In line with that, autophagy induction (by fasting or caloric restriction) resulted in ATP release and improved chemotherapy "efficacy" by decreasing TME infiltration by Treg (78).

CD39, an ectonucleotidase, also influences the extracellular ATP concentration by converting extracellular ATP into adenosine. CD39 overexpression was observed in *Atg5* deficient tumors, leading to the attraction of Treg expressing adenosinergic receptors to the TME (48). Indeed, an enhanced number of initial tumor foci and increased Treg infiltration were observed in autophagy-deficient tumors in the KRasG12D-driven lung cancer mouse model (48). In contrast, similar T cell infiltration and function in autophagy-deficient (due to inhibition of autophagy essential genes *Atg7* and *Atg12*) or competent tumors were observed in the B16 melanoma mouse model, even after Doxorubicin treatment (10). These data suggest that autophagy-dependent immune modulation may be

specific to the clinical context and time (10). However, the complexity of autophagy role in carcinogenesis can be seen in the KRasG12D-driven lung cancer mouse model, because mice with autophagy-deficient-tumors presented a prolonged survival and reduced malignant progression of adenomas to adenocarcinomas, in a TP53 dependent manner. Although the autophagy modulation needs to be investigated in each case, these data indicated that tumor-specific loss of *Atg5* favored Treg TILs. Thus, autophagy-deficient tumors (or with CD39 overexpression) treated with ICD inducer chemotherapy agents did not recruit effector cells and it possibly contributed to chemotherapy (CT) resistance (48).

Indeed, there is evidence that autophagy may play a role in CT resistance in pancreatic cancer patients. A clinical trial to investigate if autophagy inhibition by HCQ (Hydroxychloroquine sulfate) improved overall survival of metastatic pancreatic cancer patients treated with gemcitabine hydrochloride and nab-paclitaxel was performed (79). Autophagy inhibition did not improve 1-year patients' overall survival, but there was an improvement in the overall response rate in HCQ treated patients, indicating its role in the locally advanced setting, supporting the need for more research and biomarkers to drive this therapeutic option. Likewise, the treatment of bladder cancer cells (J82 and T24) with enzalutamide, an anti-androgen receptor drug, resulted in cytoplasmatic autophagosomes accumulation, increased expression of autophagy-related genes (*AMPK*, *ATG5*, *LC3B*, *ULK1*, and *LC3-II*) and had no effect on apoptosis and proliferation rates. However, the treatment with a combination of enzalutamide and autophagy inhibitors (CQ, 3-methyladenine, and bafilomycin A1) impaired tumor growth, indicating that the combined treatment may be a potential strategy to avoid enzalutamide-resistant bladder cancer (80). A similar result was exhibited in docetaxel resistant prostate cancer cell lines (PC3-DR and VCaP-DR): these cells present enhanced autophagy activity through the overexpression of Forkhead box protein M1 (*FOXM1*). Thus, the knockout of *ATG7*, *Beclin-1*, or treatment with CQ restored the antitumor effect of docetaxel, demonstrating that either autophagy or *FOXM1* may be potential targets for combined therapies with docetaxel to treat metastatic prostate cancer patients (81). On the other hand, new therapies may be used to activate autophagy, improving the efficacy of conventional treatment, such as CT. Oxaliplatin-induced ICD was not sufficient to completely eliminate both breast and colorectal tumors (82, 83). Oxaliplatin treatment together with a nanoparticle in CT26 tumor-bearing mice led to autophagy activation on tumor cells, improving antigen presentation, and consequently tumor cell death (83).

The role of autophagy in other cells beyond the tumor ones was investigated to elucidate the hypothesis that autophagy competence in the immune system would contribute to the reduction of tumor growth by ICD-inducing CT. However, the growth of autophagy competent tumors was the same in wild type and partially autophagy-deficient (*Becn1*± or *Atg4b*$^{-/-}$) mice, although the MTX toxicity was higher on *Atg4b*$^{-/-}$ mice (84). These results pointed to an autophagy role in tumor cells influencing anticancer immune responses induced by CT. Nonetheless, a similar T cell profile was observed in the B16 melanoma mouse model, both in tumor-specific autophagy inhibition treated with Doxorubicin (10) and in systemic inhibition of autophagy with CQ and quinacrine, suggesting that host autophagy competence did not influence the efficacy of ICD-inducing CT in this model (10).

Response to radiotherapy may also be influenced by autophagy. Radiation therapy induced MHC-I expression in NSCLC cell lines A549 and H1975 in parallel with an increase in the LC3-II/LC3-I ratio, while p62 detection decreased (85). Treatment of both cell lines with autophagy inhibitor CQ after radiation resulted in decreased MHC-I expression, indicating that radiation-induced MHC-I expression and CD8$^+$ T cell infiltration were dependent on autophagy (85). It was also demonstrated that imiquimod (TLR7 agonist) activated oxidative stress, inducing autophagy, and sensibilization of melanoma cells to γ-ionizing radiation (86). Mouse treatment with 3-MA, an autophagy inhibitor, after radiation restored the B16F10 or B16F1 tumor growth. The combined imiquimod and radiation therapy increased IFN-γ and TNF-α secreting CD8$^+$ T lymphocytes and decreased Treg and MDSCs in the TME (86), indicating the influence of autophagy in the regulation of therapy-induced immune responses.

Autophagy Improves New Therapeutic Strategies, by Modulating TME Components

Recently, immunotherapy has emerged as a therapeutic option able to elicit high rates of durable anti-tumor responses in cancer patients, including the ones with previously refractory responses to CRT (87). Immunotherapy aims to activate and recruit immune cells to the TME to target transformed cells. The best results have been achieved in patients with immunogenic tumors, which express high levels of neoantigens, such as metastatic melanoma. Even so, a proportion of patients with no clinical benefit after immune checkpoint blockade therapy has been reported in several studies, and autophagy may play a role in this resistance to therapy. A set of melanoma-associated antigen (MAGE) cancer-germline antigens was identified as a predictor of CTLA-4 blockade resistance in melanoma patients (88). Interestingly, autophagy markers, including LC3B, were enriched in MAGE negative tumors, suggesting that autophagy suppression (in MAGE positive tumors) may contribute to resistance to CTLA-4 inhibitor therapy (88). Another autophagy mechanism that influences the TME was observed in head and neck squamous cell carcinoma (HNSCC). Despite the high immune cell infiltration in HNSCC, response rates to immune checkpoint blockade therapy are low. It was identified that the SOX2 oncoprotein elicited an autophagy-dependent degradation of STING, which mediates IFN-I activation, important for Th1 chemokines production and M1-like macrophage polarization (89). In mice with HNSCC, the immunosuppressive TME could be reversed by vaccination with nanosatellite SatVax, which enhanced the potency of STING agonist and delivered high-density tumor antigens, improving tumor-specific T cell infiltration. Associated with anti-PD-L1 therapy, it could prevent the CD8$^+$ T cell exhaustion (the therapy expanded CTL effectors and reduces the CD8$^+$ T cells exhausted) (89).

Along with that, autophagy-deficient 4T1 cells, through *Atg5* or *Beclin1* depletion with specific single guide(sg)RNA, generated larger tumors with reduced $CD4^+$ and $CD8^+$ TILs and IFNγ^+ T cells in Balb/c mice, when compared to autophagy competent 4T1 cells. Moreover, the antitumor effect of the anti-PD1 antibody was limited in autophagy-deficient tumors, while a significant reduction in tumor volume and increased cytotoxic activity of TILs was observed in the control group (90). Similarly, a combination of anti-PD1 immunotherapy with anti-angiogenic endostatin, Endostar, promoted the activation of autophagy pathway PI3K/AKT/mTOR in LLC-bearing mice. Combined therapy suppressed tumor growth and modulated TME, decreasing IL-17 and TGFβ1 while reducing infiltrated MDSCs and reversing $CD8^+$ T cell suppression (91). These results indicated that the complex relationship between autophagy and TME components may be important not only to the modulation of immune responses but also to define treatment efficacy and resistance.

In addition to immunotherapy, a new therapeutic approach using RP-182, a synthetic peptide analog to naturally occurring antimicrobial peptides, triggered a conformational switch in the mannose receptor CD206, M2 macrophage marker. It resulted in endocytosis, autophagy, and apoptosis of these cells and also a shift toward an M1 phenotype in the remaining cells. This treatment enriched M1-like macrophages in TME and increased the antitumor immune response in the pancreatic cancer animal model, as well as CT-26 and B16 models (92).

CONCLUSIONS

In conclusion, we described data suggesting that autophagic activity plays a dual role in cancer development and progression, modulating TME in many different ways, that can either help or inhibit tumor development, as shown in **Figure 2**. DC-autophagy and TME-induced autophagy are usually associated with better antitumor responses and improvement of antigen presentation and cytotoxic activity, inhibiting regulatory T lymphocytes and MDSCs. However, myeloid cells and tumor cells autophagy seem to have the opposite effect. It improves immunosuppressive TME, through the recruitment of MDSCs and M2-like macrophage polarization, leading to tumor progression and worst prognosis. The influence of autophagy also reaches cancer treatment. The activation of autophagy pathways modulates TME by inducing macrophage polarization (M1-like phenotype), reducing $CD8^+$ T cell exhaustion and Tregs infiltration. Therefore, targeting autophagy could improve ICD-induced by conventional and non-conventional therapies.

AUTHOR CONTRIBUTIONS

This review was drafted by AS, LG, APL, and PA-S, then critically revised by PA-S. Figures were elaborated by AS and PA-S. All authors contributed to the article and approved the submitted version.

FUNDING

We thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the scholarship provided to LG (141955/2020-1) and fellowship to AL (307841/2018-9), and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarship provided to AS (88882.382114/2019-01). This work was partially funded by LIGH-FUNPAR Alliance.

REFERENCES

1. Dighe AS, Richards E, Old LJ, Schreiber RD. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFNγ receptors. *Immunity* (1994) 1:447–56. doi: 10.1016/1074-7613(94)90087-6
2. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, et al. IFNgamma and lympohcytes prevent primary tomour development and shape tomour immunogenicity. *Nature* (2001) 410:1107–11. doi: 10.1038/35074122
3. Dunn GP, Old LJ, Schreiber RD. The Three Es of Cancer Immunoediting. *Annu Rev Immunol* (2004) 22:329–60. doi: 10.1146/annurev.immunol.22.012703.104803
4. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: Integrating immunity's roles in cancer suppression and promotion. *Science (80-)* (2011) 331:1565–70. doi: 10.1126/science.1203486
5. Roma-Rodrigues C, Mendes R, Baptista PV, Fernandes AR. Targeting tumor microenvironment for cancer therapy. *Int J Mol Sci* (2019) 20:840. doi: 10.3390/ijms20040840
6. Jarosz-Biej M, Smolarczyk R, Cichoń T, Kułach N. Tumor microenvironment as a "game changer" in cancer radiotherapy. *Int J Mol Sci* (2019) 20:18–20. doi: 10.3390/ijms20133212
7. Deter RL, Baudhuin P, De Duve C. Participation of lysosomes in cellular autophagy induced in rat liver by glucagon. *J Cell Biol* (1967) 35:C11–6. doi: 10.1083/jcb.35.2.c11
8. Tsukada M. Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae. *FEBS Lett* (1993) 333:169–74. doi: 10.1016/0014-5793(93)80398-E
9. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* (1999) 402:672–6. doi: 10.1038/45257
10. Starobinets H, Ye J, Broz M, Barry K, Goldsmith J, Marsh T, et al. Antitumor adaptive immunity remains intact following inhibition of autophagy and antimalarial treatment. *J Clin Invest* (2016) 126:4417–29. doi: 10.1172/JCI85705
11. Yamamoto K, Venida A, Yano J, Biancur DE, Kakiuchi M, Gupta S, et al. Autophagy promotes immune evasion of pancreatic cancer by degrading MHC-I. *Nature* (2020) 581:100–5. doi: 10.1038/s41586-020-2229-5
12. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. *Cell* (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
13. Garner H, de Visser KE. Immune crosstalk in cancer progression and metastatic spread: a complex conversation. *Nat Rev Immunol* (2020) 20 (8):483–97. doi: 10.1038/s41577-019-0271-z
14. Engels EA, Pfeiffer RM, Goedert JJ, Virgo P, Mcneel TS, Scoppa SM, et al. Trends in cancer risk among people with AIDS in the United States 1980 – 2002. *AIDS* (2006) 20:1645–54. doi: 10.1097/01.aids.0000238411.75324.59
15. Franzetti M, Ricci E, Bonfanti P. The Pattern of Non-AIDS-defining Cancers in the HIV Population: Epi- demiology, Risk Factors and Prognosis. A Review. *Curr HIV Res* (2019) 17:1–12. doi: 10.2174/1570162X17666190327153038

16. Patel SJ, Sanjana NE, Kishton RJ, Eidizadeh A, Vodnala SK, Cam M, et al. Identification of essential genes for cancer immunotherapy. *Nature* (2017) 548 (7669):537–42. doi: 10.1038/nature23477
17. Zhang C, Liu Y. Targeting NK Cell Checkpoint Receptors or Molecules for Cancer Immunotherapy. *Front Immunol* (2020) 11:1295. doi: 10.3389/fimmu.2020.01295
18. Knochelmann HM, Dwyer CJ, Smith AS, Bowers JS, Wyatt MM, Nelson MH, et al. IL-6 fuels durable memory for Th17 cell-mediated responses to tumors. *Cancer Res* (2020) 80(18):3920–32. doi: 10.1158/0008-5472.can-19-3685
19. Minetto P, Guolo F, Pesce S, Greppi M, Obino V, Ferretti E, et al. Harnessing NK Cells for Cancer Treatment. *Front Immunol* (2019) 10:2836. doi: 10.3389/fimmu.2019.02836
20. Martinez-Lostao L, Anel A, Pardo J. How Do Cytotoxic Lymphocytes Kill Cancer Cells? *Clin Cancer Res* (2015) 21:5047–56. doi: 10.1158/1078-0432.CCR-15-0685
21. Tay RE, Richardson EK, Toh HC. Revisiting the role of CD4 + T cells in cancer immunotherapy — new insights into old paradigms. *Cancer Gene Ther* (2020). doi: 10.1038/s41417-020-0183-x
22. Park J, Kim E, Kwon H, Hwang E, Namkoong S, Um S. Inactivation of Interferon Regulatory Factor-1 Tumor Suppressor Protein by HPV E7 Oncoprotein. *J Biol Chem* (2000) 275:6764–9. doi: 10.1074/jbc.275.10.6764
23. Aricò E, Castiello L, Capone I, Gabriele L, Belardelli F. Type i interferons and cancer: An evolving story demanding novel clinical applications. *Cancers (Basel)* (2019) 11(12):1943. doi: 10.3390/cancers11121943
24. Bussey KA, Brinkmann MM. Strategies for immune evasion by human tumor viruses. *Curr Opin Virol* (2018) 32:30–9. doi: 10.1016/j.coviro.2018.08.015
25. Keerthivasan S, Aghajani K, Dose M, Molinero L, Khan W, Venkatesvaran V, et al. Wnt/β-catenin signaling in T-cells drives epigenetic imprinting of pro-inflammatory properties and promotes colitis and colon cancer. *Sci Transl Med* (2014) 6(225):225ra28. doi: 10.1126/scitranslmed.3007607.Wnt/
26. Kroemer G, Pouyssegur J. Tumor Cell Metabolism: Cancer's Achilles' Heel. *Cancer Cell* (2008) 13(6):472–82. doi: 10.1016/j.ccr.2008.05.005
27. Domblides C, Lartigue L, Faustin B. Control of the Antitumor Immune Response by Cancer Metabolism. *Cells* (2019) 8(2):104. doi: 10.3390/cells8020104
28. Mezrich JD, Fechner JH, Zhang X, Johnson BP, Burlingham WJ, Bradfield CA. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. *J Immunol* (2011) 185:3190–8. doi: 10.4049/jimmunol.0903670.AN
29. Hornyák L, Dobos N, Koncz G, Karányi Z, Páll D, Szabó Z, et al. The Role of indoleamine-2, 3- Dioxygenase in Cancer Development, Diagnostics, and Therapy. *Front Immunol* (2018) 9:151. doi: 10.3389/fimmu.2018.00151
30. Alspach E, Lussier DM, Schreiber RD. Interferon γ and its important roles in promoting and inhibiting spontaneous and therapeutic cancer immunity. *Cold Spring Harb Perspect Biol* (2019) 11(3):a028480. doi: 10.1101/cshperspect.a028480
31. Bally APR, Austin JW, Boss JM. Genetic and epigenetic regulation of PD-1 expression. *J Immunol* (2017) 196:2431–7. doi: 10.4049/jimmunol.1502643.Genetic
32. Jansen CS, Prokhnevska N, Master VA, Sanda MG, Carlisle JW, Bilen MA, et al. An intra-tumoral niche maintains and differentiates stem-like CD8 T cells. *Nature* (2019) 576:465–73. doi: 10.1038/s41586-019-1836-5
33. Kurachi M. CD8+ T cell exhaustion. *Semin Immunopathol* (2019) 41:327–37. doi: 10.1007/s00281-019-00744-5
34. Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM, et al. Trans-endocytosis of CD80 and CD86 : a molecular basis for the cell extrinsic function of CTLA-4. *Science (80-)* (2011) 332:600–3. doi: 10.1126/science.1202947.Trans-endocytosis
35. Genard G, Lucas S, Michiels C. Reprogramming of tumor-associated macrophages with anticancer therapies: Radiotherapy versus chemo- and immunotherapies. *Front Immunol* (2017) 8:828. doi: 10.3389/fimmu.2017.00828
36. Duve C D, Pressman BC, Gianetto R, Wattiaux R, Appelmans F. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem J* (1955) 60:604–17. doi: 10.1042/bj0600604
37. Moscat J, Diaz-Meco MT. p62 at the Crossroads of Autophagy, Apoptosis, and Cancer. *Cell* (2009) 137(6):1001–4. doi: 10.1016/j.cell.2009.05.023
38. Glick D, Barth S, Macleod KF. Autophagy: Cellular and molecular mechanisms. *J Pathol* (2010) 221(1):3–12. doi: 10.1002/path.2697
39. Sahu R, Kaushik S, Clement CC, Cannizzo ES, Scharf B, Follenzi A, et al. Microautophagy of Cytosolic Proteins by Late Endosomes. *Dev Cell* (2011) 20:131–9. doi: 10.1016/j.devcel.2010.12.003
40. Cuervo AM, Dice JF. A receptor for the selective uptake and degradation of proteins by lysosomes. *Science (80-)* (1996) 273:501–3. doi: 10.1126/science.273.5274.501
41. Bellot G, Garcia-Medina R, Gounon P, Chiche J, Roux D, Pouysségur J, et al. Hypoxia-Induced Autophagy Is Mediated through Hypoxia-Inducible Factor Induction of BNIP3 and BNIP3L via Their BH3 Domains. *Mol Cell Biol* (2009) 29:2570–81. doi: 10.1128/mcb.00166-09
42. Egan DF, Shackelford DB, Mihaylova MM, Gelino SR, Kohnz RA, Mair W, et al. and SR. Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. *Science (80-)* (2011) 28:456–61. doi: 10.1126/science.1196371
43. Papandreou I, Lim AL, Laderoute K, Denko NC. Hypoxia signals autophagy in tumor cells via AMPK activity, independent of HIF-1, BNIP3, and BNIP3L. *Cell Death Differ* (2008) 15:1572–81. doi: 10.1038/cdd.2008.84
44. Yang Y, Klionsky DJ. Autophagy and disease: unanswered questions. *Cell Death Differ* (2020) 27(3):858–87. doi: 10.1038/s41418-019-0480-9
45. White E, Mehnert JM, Chan CS. Autophagy, Metabolism, and Cancer. *Clin Cancer Res* (2015) 21:5037–46. doi: 10.1158/1078-0432.CCR-15-0490
46. Ireland JM, Unanue ER. Autophagy in antigen-presenting cells results in presentation of citrullinated peptides to CD4 T cells. *J Exp Med* (2011) 208:2625–32. doi: 10.1084/jem.20110640
47. Li Y, Hahn T, Garrison K, Cui ZH, Thorburn A, Thorburn J, et al. The vitamin E analogue α-TEA stimulates tumor autophagy and enhances antigen cross-presentation. *Cancer Res* (2012) 72:3535–45. doi: 10.1158/0008-5472.CAN-11-3103
48. Rao S, Tortola L, Perlot T, Wirnsberger G, Novatchkova M, Nitsch R, et al. A dual role for autophagy in a murine model of lung cancer. *Nat Commun* (2014) 5:3056. doi: 10.1038/ncomms4056
49. Wang Y, Lin YX, Wang J, Qiao SL, Liu YY, Dong WQ, et al. In Situ Manipulation of Dendritic Cells by an Autophagy-Regulative Nanoactivator Enables Effective Cancer Immunotherapy. *ACS Nano* (2019) 13:7568–77. doi: 10.1021/acsnano.9b00143
50. Khateri M, Abdoli A, Motevalli F, Fotouhi F, Bolhassani A, Arashkia A, et al. Evaluation of autophagy induction on HEV 239 vaccine immune response in a mouse model. *IUBMB Life* (2018) 70:207–14. doi: 10.1002/iub.1719
51. Munz C. Enhancing immunity through autophagy. *Annu Rev Immunol* (2009) 27:423–49. doi: 10.1146/annurev.immunol.021908.132537 10.1146/annurev.immunol.021908.132537.
52. Reeves E, James E. Antigen processing and immune regulation in the response to tumours. *Immunology* (2017) 150:16–24. doi: 10.1111/imm.12675
53. Münz C. Autophagy Beyond Intracellular MHC Class II Antigen Presentation. *Trends Immunol* (2016) 37:755–63. doi: 10.1016/j.it.2016.08.017
54. Dendrou CA, Petersen J, Rossjohn J, Fugger L. HLA variation and disease. *Nat Rev Immunol* (2018) 18:325–39. doi: 10.1038/nri.2017.143
55. Tumeh PC, Hellmann MD, Hamid O, Tsai KK, Loo KL, Gubens MA, et al. Liver metastasis and treatment outcome with anti-PD-1 monoclonal antibody in patients with melanoma and NSCLC. *Cancer Immunol Res* (2017) 5:417–24. doi: 10.1158/2326-6066.CIR-16-0325
56. Takahashi Y, Yasui T, Minami K, Tamari K, Otani K, Seo Y, et al. Radiation enhances the efficacy of antitumor immunotherapy with an immunocomplex of interleukin-2 and its monoclonal antibody. *Anticancer Res* (2017) 37:6799–806. doi: 10.21873/anticanres.12140
57. Peggs KS, Segal NH, Allison JP. Targeting Immunosupportive Cancer Therapies: Accentuate the Positive, Eliminate the Negative. *Cancer Cell* (2007) 12:192–9. doi: 10.1016/j.ccr.2007.08.023
58. Brentville VA, Metheringham RL, Daniels I, Atabani S, Symonds P, Cook KW, et al. Combination vaccine based on citrullinated vimentin and enolase peptides induces potent CD4-mediated anti-tumor responses. *J Immunother Cancer* (2020) 8:6–9. doi: 10.1136/jitc-2020-000560
59. Baghdadi M, Yoneda A, Yamashina T, Nagao H, Komohara Y, Nagai S, et al. TIM-4 Glycoprotein-Mediated Degradation of Dying Tumor Cells by Autophagy Leads to Reduced Antigen Presentation and Increased Immune Tolerance. *Immunity* (2013) 39:1070–81. doi: 10.1016/j.immuni.2013.09.014

60. Ramirez JAZ, Romagnoli GG, Falasco BF, Gorgulho CM, Sanzochi Fogolin C, dos Santos DC, et al. Blocking drug-induced autophagy with chloroquine in HCT-116 colon cancer cells enhances DC maturation and T cell responses induced by tumor cell lysate. *Int Immunopharmacol* (2020) 84:106495. doi: 10.1016/j.intimp.2020.106495
61. Tu SP, Quante M, Bhagat G, Takaishi S, Cui G, Yang XD, et al. IFN- g Inhibits Gastric Carcinogenesis by Inducing Epithelial Cell Autophagy and T-cell Apoptosis. *Cancer Res* (2011) 71:4247–59. doi: 10.1158/0008-5472.CAN-10-4009
62. Vodnala SK, Eil R, Kishton RJ, Sukumar M, Yamamoto TN, Ha N, et al. T cell stemness and dysfunction in tumors are triggered by a common mechanism. *Science (80-)* (2019) 363:1–12. doi: 10.1126/science.aau0135
63. Cunha LD, Yang M, Carter R, Guy C, Harris L, Crawford C, et al. LC3-associated phagocytosis in myeloid cells promotes tumor immune tolerance. *Cell* (2019) 175:429–41. doi: 10.1016/j.cell.2018.08.061.LC3-associated
64. Alissafi T, Hatzioannou A, Mintzas K, Barouni RM, Banos A, Sormendi S, et al. Autophagy orchestrates the regulatory program of tumor-associated myeloid-derived suppressor cells. *J Clin Invest* (2018) 128:3840–52. doi: 10.1172/JCI120888
65. Baginska J, Viry E, Berchem G, Poli A, Noman MZ, Van Moer K, et al. Granzyme B degradation by autophagy decreases tumor cell susceptibility to natural killer-mediated lysis under hypoxia. *Proc Natl Acad Sci U S A* (2013) 110:17450–5. doi: 10.1073/pnas.1304790110
66. Mgrditchian T, Arakelian T, Paggetti J, Noman MZ, Viry E, Moussay E, et al. Targeting autophagy inhibits melanoma growth by enhancing NK cells infiltration in a CCL5-dependent manner. *Proc Natl Acad Sci U S A* (2017) 114:E9271–9. doi: 10.1073/pnas.1703921114
67. Valdor R, García-Bernal D, Riquelme D, Martinez CM, Moraleda JM, Cuervo AM, et al. Glioblastoma ablates pericytes antitumor immune function through aberrant up-regulation of chaperone-mediated autophagy. *Proc Natl Acad Sci U S A* (2019) 116:20655–65. doi: 10.1073/pnas.1903542116
68. Li W, Tanikawa T, Kryczek I, Xia H, Li G, Wu K, et al. Aerobic Glycolysis Controls Myeloid-Derived Suppressor Cells and Tumor Immunity via a Specific Article Aerobic Glycolysis Controls Myeloid-Derived Suppressor Cells and Tumor Immunity via a Specific CEBPB Isoform in Triple-Negative Breast Cancer. *Cell Metab* (2018) 28:87–103. doi: 10.1016/j.cmet.2018.04.022
69. DeVorkin L, Pavey N, Carleton G, Comber A, Ho C, Lim J, et al. Autophagy Regulation of Metabolism Is Required for CD8 + T Cell Anti-tumor Immunity. *Cell Rep* (2019) 27:502–513.e5. doi: 10.1016/j.celrep.2019.03.037
70. Sciarretta F, Fulci C, Palumbo C, Rotili D, Tentori L, Graziani G, et al. Effects of Glutathione Transferase-Targeting Nitrobenzoxadiazole Compounds in Relation to PD-L1 Status in Human Melanoma Cells. *Chemotherapy* (2019) 64:138–45. doi: 10.1159/000503339
71. Galluzzi L, Pietrocola F, Bravo-San Pedro JM, Amaravadi RK, Baehrecke EH, Cecconi F, et al. Autophagy in malignant transformation and cancer progression. *EMBO J* (2015) 34:856–80. doi: 10.15252/embj.201490784
72. Casares N, Pequignot MO, Tesniere A, Ghiringhelli F, Roux S, Chaput N, et al. Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *J Exp Med* (2005) 202:1691–701. doi: 10.1084/jem.20050915
73. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med* (2007) 13:54–61. doi: 10.1038/nm1523
74. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med* (2007) 13:1050–9. doi: 10.1038/nm1622
75. Saenz R, Futalan D, Leutenez L, Eekhout F, Fecteau JF, Sundelius S, et al. TLR4-dependent activation of dendritic cells by an HMGB1-derived peptide adjuvant. *J Transl Med* (2014) 12:1–11. doi: 10.1186/1479-5876-12-211
76. Michaud M, Martins I, Sukkurwala AQ, Adjemian S, Ma Y, Pellegatti P, et al. Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. *Science (80-)* (2011) 334:1573–7. doi: 10.1126/science.1208347
77. Ma Y, Adjemian S, Mattarollo SR, Yamazaki T, Aymeric L, Yang H, et al. Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells. *Immunity* (2013) 38:729–41. doi: 10.1016/j.immuni.2013.03.003
78. Pietrocola F, Pol J, Vacchelli E, Rao S, Enot DP, Baracco EE, et al. Caloric Restriction Mimetics Enhance Anticancer Immunosurveillance. *Cancer Cell* (2016) 30:147–60. doi: 10.1016/j.ccell.2016.05.016
79. Karasic TB, O'Hara MH, Loaiza-Bonilla A, Reiss KA, Teitelbaum UR, Borazanci E, et al. Effect of Gemcitabine and nab-Paclitaxel with or Without Hydroxychloroquine on Patients with Advanced Pancreatic Cancer: A Phase 2 Randomized Clinical Trial. *JAMA Oncol* (2019) 5:993–8. doi: 10.1001/jamaoncol.2019.0684
80. Quan Y, Lei H, Wahafu W, Liu Y, Ping H, Zhang X. Inhibition of autophagy enhances the anticancer effect of enzalutamide on bladder cancer. *BioMed Pharmacother* (2019) 120:109490. doi: 10.1016/j.biopha.2019.109490
81. Lin JZ, Wang WW, Hu TT, Zhu GY, Li LN, Zhang CY, et al. FOXM1 contributes to docetaxel resistance in castration-resistant prostate cancer by inducing AMPK/mTOR-mediated autophagy. *Cancer Lett* (2020) 469:481–9. doi: 10.1016/j.canlet.2019.11.014
82. Feng B, Zhou F, Hou B, Wang D, Wang T, Fu Y, et al. Binary Cooperative Prodrug Nanoparticles Improve Immunotherapy by Synergistically Modulating Immune Tumor Microenvironment. *Adv Mater* (2018) 30:e1803001. doi: 10.1002/adma.201803001
83. Wang X, Li M, Ren K, Xia C, Li J, Yu Q, et al. On-Demand Autophagy Cascade Amplification Nanoparticles Precisely Enhanced Oxaliplatin-Induced Cancer Immunotherapy. *Adv Mater* (2020) 32:e2002160. doi: 10.1002/adma.202002160
84. Castoldi F, Vacchelli E, Zitvogel L, Maiuri MC, Pietrocola F, Kroemer G. Systemic autophagy in the therapeutic response to anthracycline-based chemotherapy. *Oncoimmunology* (2019) 8:1–6. doi: 10.1080/2162402X.2018.1498285
85. Zeng H, Zhang W, Gong Y, Xie C. Radiotherapy activates autophagy to increase CD8+ T cell infiltration by modulating major histocompatibility complex class-I expression in non-small cell lung cancer. *J Int Med Res* (2019) 47:3818–30. doi: 10.1177/0300060519855595
86. Cho JH, Lee HJ, Ko HJ, Yoon BI, Choe J, Kim KC, et al. The TLR7 agonist imiquimod induces anti-cancer effects via autophagic cell death and enhances anti-tumoral and systemic immunity during radiotherapy for melanoma. *Oncotarget* (2017) 8:24932–48. doi: 10.18632/oncotarget.15326
87. Robert C, Marabelle A, Herrscher H, Caramella C, Rouby P, Fizazi K, et al. Immunotherapy discontinuation — how, and when? Data from melanoma as a paradigm. *Nat Rev Clin Oncol* (2020) 17:707–15. doi: 10.1038/s41571-020-0399-6
88. Shukla SA, Bachireddy P, Schilling B, Galonska C, Zhan Q, Bango C, et al. Cancer-germline antigen expression discriminates clinical outcome to CTLA-4 blockade. *Cell* (2018) 173:624–33. doi: 10.1016/j.cell.2018.03.026
89. Tan YS, Sansanaphongpricha K, Xie Y, Christopher R, Luo X, Heath BR, et al. Mitigating SOX2-potentiated immune escape of Head and Neck Squamous Cell Carcinoma with a STING-inducing nanosatellite vaccine. *Clin Cancer Res* (2018) 24:4242–55. doi: 10.1158/1078-0432.CCR-17-2807
90. Li ZL, Zhang HL, Huang Y, Huang JH, Sun P, Zhou NN, et al. Autophagy deficiency promotes triple-negative breast cancer resistance to T cell-mediated cytotoxicity by blocking tenascin-C degradation. *Nat Commun* (2020) 11:3806. doi: 10.1038/s41467-020-17395-y
91. Wu J, Zhao X, Sun Q, Jiang Y, Zhang W, Luo J, et al. Synergic effect of PD-1 blockade and endostar on the PI3K/AKT/mTOR-mediated autophagy and angiogenesis in Lewis lung carcinoma mouse model. *BioMed Pharmacother* (2020) 125:109746. doi: 10.1016/j.biopha.2019.109746
92. Jaynes JM, Sable R, Ronzetti M, Bautista W, Knotts Z, Abisoye-Ogunniyan A, et al. Mannose receptor (CD206) activation in tumor-associated macrophages enhances adaptive and innate antitumor immune responses. *Sci Transl Med* (2020) 12:1–20. doi: 10.1126/scitranslmed.aax6337

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 de Souza, Gonçalves, Lepique and de Araujo-Souza. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Oncology

REVIEW
published: 07 December 2020
doi: 10.3389/fonc.2020.605314

Deciphering the Role of the Coagulation Cascade and Autophagy in Cancer-Related Thrombosis and Metastasis

Charlotte Nicole Hill[1,2,3], *Maria Paz Hernández-Cáceres*[1], *Catalina Asencio*[1,3], *Begoña Torres*[1], *Benjamin Solis*[1] *and Gareth I. Owen*[1,2,3,4*]

[1] Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, Santiago, Chile, [2] Advanced Center for Chronic Diseases (ACCDiS), Santiago, Chile, [3] Millennium Institute on Immunology and Immunotherapy, Santiago, Chile, [4] Faculty of Medicine, Pontificia Universidad Católica de Chile, Santiago, Chile

Thrombotic complications are the second leading cause of death among oncology patients worldwide. Enhanced thrombogenesis has multiple origins and may result from a deregulation of megakaryocyte platelet production in the bone marrow, the synthesis of coagulation factors in the liver, and coagulation factor signaling upon cancer and the tumor microenvironment (TME). While a hypercoagulable state has been attributed to factors such as thrombocytosis, enhanced platelet aggregation and Tissue Factor (TF) expression on cancer cells, further reports have suggested that coagulation factors can enhance metastasis through increased endothelial-cancer cell adhesion and enhanced endothelial cell activation. Autophagy is highly associated with cancer survival as a double-edged sword, as can both inhibit and promote cancer progression. In this review, we shall dissect the crosstalk between the coagulation cascade and autophagic pathway and its possible role in metastasis and cancer-associated thrombosis formation. The signaling of the coagulation cascade through the autophagic pathway within the hematopoietic stem cells, the endothelial cell and the cancer cell are discussed. Relevant to the coagulation cascade, we also examine the role of autophagy-related pathways in cancer treatment. In this review, we aim to bring to light possible new areas of cancer investigation and elucidate strategies for future therapeutic intervention.

Keywords: cancer, metastasis, coagulation, autophagy, cancer-associated thrombosis, megakaryopoeiesis and thrombopoiesis, protease (proteinase)-activated receptor

OPEN ACCESS

Edited by:
Claudio Marcelo Fader, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

Reviewed by:
Elena Ciaglia, University of Salerno, Italy
Mariano A. Ostuni, Université Paris Diderot, France

***Correspondence:**
Gareth I. Owen
gowen@bio.puc.cl

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *11 September 2020*
Accepted: *29 October 2020*
Published: *07 December 2020*

Citation:
Hill CN, Hernández-Cáceres MP, Asencio C, Torres B, Solis B and Owen GI (2020) Deciphering the Role of the Coagulation Cascade and Autophagy in Cancer-Related Thrombosis and Metastasis. Front. Oncol. 10:605314. doi: 10.3389/fonc.2020.605314

INTRODUCTION

While the precise role of the extrinsic coagulation cascade in the pathophysiology of cancer progression is still largely unknown, a hypercoagulable state has been intimately linked to cancer progression for more than a century (1). Cancer-associated thrombosis (CAT), or Trousseau's syndrome, is attributed as the second leading cause of cancer patient death after organ failure upon metastatic disease (2, 3). High D-dimer levels, a product of coagulation cascade activation, are

associated with advance cancer stage and accordingly there is a high prevalence of venous thromboembolism (VTE) in stage IV cancer patients (4–6). Given the risk of VTE being 4- to 7-fold higher in the cancer patient, modulation of the coagulation system is currently an essential aspect of cancer treatment (7), with the potential to improve in the coming years. A chronic hypercoagulable state in oncologic patients may have a multifaceted role in conferring both a survival advance and dissemination potential to a bourgeoning tumor. This hypercoagulable state can be explained by several factors, including increased megakaryocyte (MK) and platelet production (**Figure 1A**), increased platelet activation and deregulation of other cells intertwined with coagulation systems such as endothelial cells (ECs) and neutrophils (**Figure 1B**).

Different cell types express Tissue Factor (TF), including cancer cells, platelets, and activated endothelial and immune cells. The antibody blocking of TF has been shown to delay the initiation of tumor formation, growth and vascularization in immunodeficient mice (8). Collectively, data suggest that oncogenic and differentiation pathways regulate TF and that it functions in tumor initiation, tumor growth, angiogenesis, and metastasis. At the molecular level, TF, a transmembrane receptor belonging to cytokine receptor family, initiates the extrinsic blood coagulation pathway once bound in a quaternary complex with activated factor VII (FVIIa) and coagulation factor X (FX) (**Figure 1C**) [reviewed in (9)]. The cleavage of FX by FVIIa gives rise to the active protease (FXa), which in turn cleaves prothrombin to thrombin. This coagulation initiation complex can also directly activate cofactor VIII and coagulation factor IX, leading to thrombin generation (9).

The principal mediators of FXa and Thrombin protease signal transduction are the protease-activated receptors (PARs). These receptors belong to the G protein-coupled receptor (GPCR) family and become activated upon proteolytic cleavage of their N-terminal domain by extracellular proteases. PAR signaling has been implicated in several inflammatory diseases, including cancer (10, 11). Mammalian genomes contain four PARs that are ubiquitous within the body. PAR1, PAR3, and PAR4 are thrombin receptors, unlike PAR2. Classically, soluble proteases that are active during vascular injury, coagulation, and inflammation are responsible for PAR activation (12). Among these proteases are plasmin, matrix metalloproteinases (MMPs), activated Protein C (aPC), FVIIa, and FXa. However, the mechanisms remain elusive (13). The proteolytic cleavage of PARs activates numerous downstream signaling pathways, including intracellular Ca^{2+} mobilization, ERK1/2, NFκB signaling pathways and the induction of cytokines such as

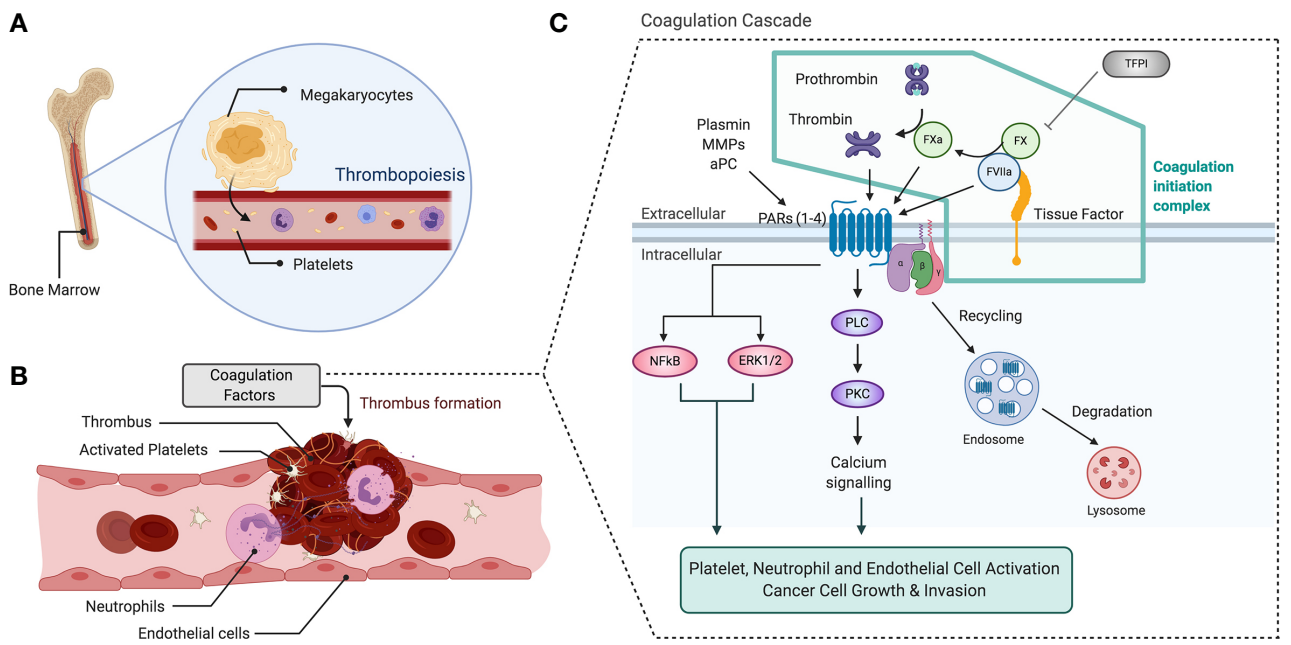


FIGURE 1 | Overview of the coagulation and clotting factor signaling. Increased levels of platelet blood counts and coagulation cascade activation are determinants of a hypercoagulable state. Platelet production (thrombopoiesis) at the bone marrow by megakaryocytes determines platelet counts **(A)**. Upon vascular damage, platelets become activated, adhere to the vascular endothelium and promote neutrophil activation and NET release **(B)**. Platelets, Neutrophils and Endothelial cells become activated and promote thrombus formation, a process mediated by the coagulation cascade activation **(C)**. TF, a transmembrane receptor, initiate the extrinsic blood coagulation pathway once bound in a quaternary complex with FVIIa, that is inhibited by TFPI. The cleavage of FX by FVIIa gives rise to FXa, which in turn cleaves prothrombin into thrombin. Thrombin, FXa and other proteases such as Plasmin, MMPs, and aPC can activate PARs, which signal through PLC-PKC-Ca^{2+} Pathway and activate other signaling pathways such as NFkB and ERK1/2, leading to platelet, neutrophil and EC activation. This pathway also promotes cancer cell growth and invasion. PAR activation leads to receptor internalization, which classically occurs through the endolyosomoal pathway. aPC, activated Protein C; ERK1/2, Extracellular signal-regulated protein kinases 1 and 2; EC, Endothelial Cell; FVIIa, activated factor VII; FX, factor X; FXa, activated FX; MMPs, matrix metalloproteases; NET, Neutrophil Extracellular Trap; NFkB, Nuclear-Factor kappa B; PAR, protease activated receptor; TFPI, Tissue Factor Pathway Inhibitor.

Interleukin-8 (IL-8) and IL-6 (14), allowing the PARs to mediate several processes in the coagulation-inflammation interplay, including those implicated in cancer progression (15, 16). Thus, PARs regulate platelet activation, assist in maintaining vascular barrier function through adhesion molecules (17, 18), and are associated with immune cell activation and migration (19–21). Upon activation, PARs are rapidly uncoupled from heterotrimeric G proteins through internalization to endosomes and then sorted to lysosomes and degraded. However, recent studies indicate that activated internalized PARs signal from endosomes through the recruitment of β-arrestins and potentially other pathways [reviewed in (10, 22)]. Interestingly, crosstalk between PAR signaling and autophagy has been described in different cell types (23–25), the physiological and pathophysiological role of this crosstalk still is an open field for investigation and discussion.

Autophagy, which comes from the Greek and means "self-eating", is a highly regulated catabolic pathway by which cytoplasmic cargo is delivered to lysosomes for degradation and recycling, in order to preserve cellular homeostasis (26–29). In cancer, autophagy is broadly recognized as a "double-edged sword", participating in both cancer suppression and promotion depending on tumor type, stage and microenvironment (30–32). Three principal types of autophagy have been identified: macroautophagy, microautophagy and chaperone-mediated autophagy (33, 34). Macroautophagy hereafter referred to as autophagy, is the most extensively characterized in cancer context and will be the focus of discussion within this review (35–37).

Autophagy involves the sequestration of cytoplasmic material, such as damaged organelles (i.e. mitochondria, endoplasmic reticulum) and protein aggregates, in a double membrane organelle called the autophagosome, which subsequently fuses to lysosomes, forming a new vesicle known as the autolysosome. Lysosomal enzymes within the autolysosome initiate the hydrolytic breakdown of their cargo (30). The resulting degradation products (i.e., sugars, amino acids, fatty acids, nucleosides/nucleotides) are transferred back to the cytoplasm for macromolecule synthesis and energy production (**Figure 2**) (38). Therefore, autophagy sustains cellular homeostasis by regulating the quality of cytoplasmic organelles. Moreover, this is also an adaptive mechanism that promotes cell survival in response to stress conditions such as nutrient deprivation, hypoxia, reactive oxygen species (ROS), DNA damage, intracellular pathogens or an increase in misfolded proteins (39, 40). Cancer cells can exploit this mechanism when exposed to metabolic, oxidative and inflammatory stress (41–43). Different "autophagy-related proteins" (ATGs) participate in the autophagic process (44), which can be divided into different stages: initiation, nucleation, elongation, autophagosome maturation and fusion with the lysosome, and finally cargo degradation followed by the release of breakdown products into the cytosol.

As schematically represented in **Figure 2**, the "initiation stage" involves the activation of the ULK1 complex, which is composed of the Unc-51-Like Kinase 1 (ULK1), the Focal adhesion-kinase family Interacting Protein of 200 kDa (FIP200), ATG101, and ATG13. The ULK1 complex integrates nutrient and energy stress signals through the activity of the Serine/Threonine kinase Mammalian Target of Rapamycin Complex 1 (mTORC1), which is known as the master negative regulator of autophagy (29). In parallel, an isolation membrane called phagophore is formed by membrane contributions from various organelles, including the endoplasmic reticulum (ER), Golgi apparatus, and mitochondria (45). Once activated, the ULK1 complex translocates to a membranous site where the phagophore is formed, where it serves as a scaffold for the recruitment of ATG proteins to the isolation membrane and thereby initiating autophagosome biogenesis (46, 47).

In the following step, referred to as the "nucleation stage" (**Figure 2**), the ULK1 complex phosphorylates the class III Phosphatidylinositol 3-Kinase (PI3K) complex, composed of Vacuolar Sorting Protein 34 (VPS34), Beclin-1 (BECN1), VPS15, and Autophagy Related 14-Like protein (ATG14L). The class III PI3K complex promotes local production of phosphatidylinositol 3-phosphate (PI3P) at the phagophore and initiates the recruitment of effector proteins to assist autophagosome formation (48, 49). ATG9, the only transmembrane protein that is part of the ATG machinery, cycles between the phagophore and the Golgi/endosomes, contributing to the recruitment of membranes for the formation and subsequent expansion of the phagophore (47, 50, 51). The sorting of ATG9 and its following transport to phagophore membranes occurs at RAB11-positive recycling endosomes (52, 53). Besides, ATG2 participates in the transfer of lipids from the ER to the phagophore leading to its expansion (54, 55).

The subsequent step is the "elongation stage" and involves the extension of the phagophore membrane (**Figure 2**). This process requires two ubiquitin-like systems: the ATG5–ATG12 system and the Microtubule-Associated Protein 1A/1B-Light Chain 3 (MAP1LC3A/B, also known as LC3)-phosphatidylethanolamine (PE) system (56). First, ATG12 is conjugated to ATG5 by the sequential activity of ATG7 and ATG10. The resulting ATG5-ATG12 complex interacts with ATG16L, leading to the formation of the multimeric complex ATG5-ATG12-ATG16L which is fundamental for LC3 lipidation (57, 58). Second, pro-LC3 is cleaved by ATG4 to form the cytosolic soluble LC3-I. Subsequently, ATG7 and ATG3 activity enable LC3-I conjugation to the lipid PE to form LC3-II, which is then recruited to the inner and outer surface of the autophagosome in an ATG5-ATG12 dependent manner (59). This process elongates and seals the phagophore leading to the subsequent formation of the autophagosome. Importantly, under basal conditions, LC3-I is uniformly distributed across the cytoplasm; however, upon autophagy induction, the lipidated form of LC3 (LC3-II) accumulates on nascent autophagosomes, and thus, represents a marker to monitor the formation of autophagosomes and autophagy-related structures (60–62). Autophagic cargo selection occurs in parallel to the processes of sensing, initiation and elongation. Cargo adaptors such as the receptor protein Sequestosome 1/p62 (SQSTM1/p62) can

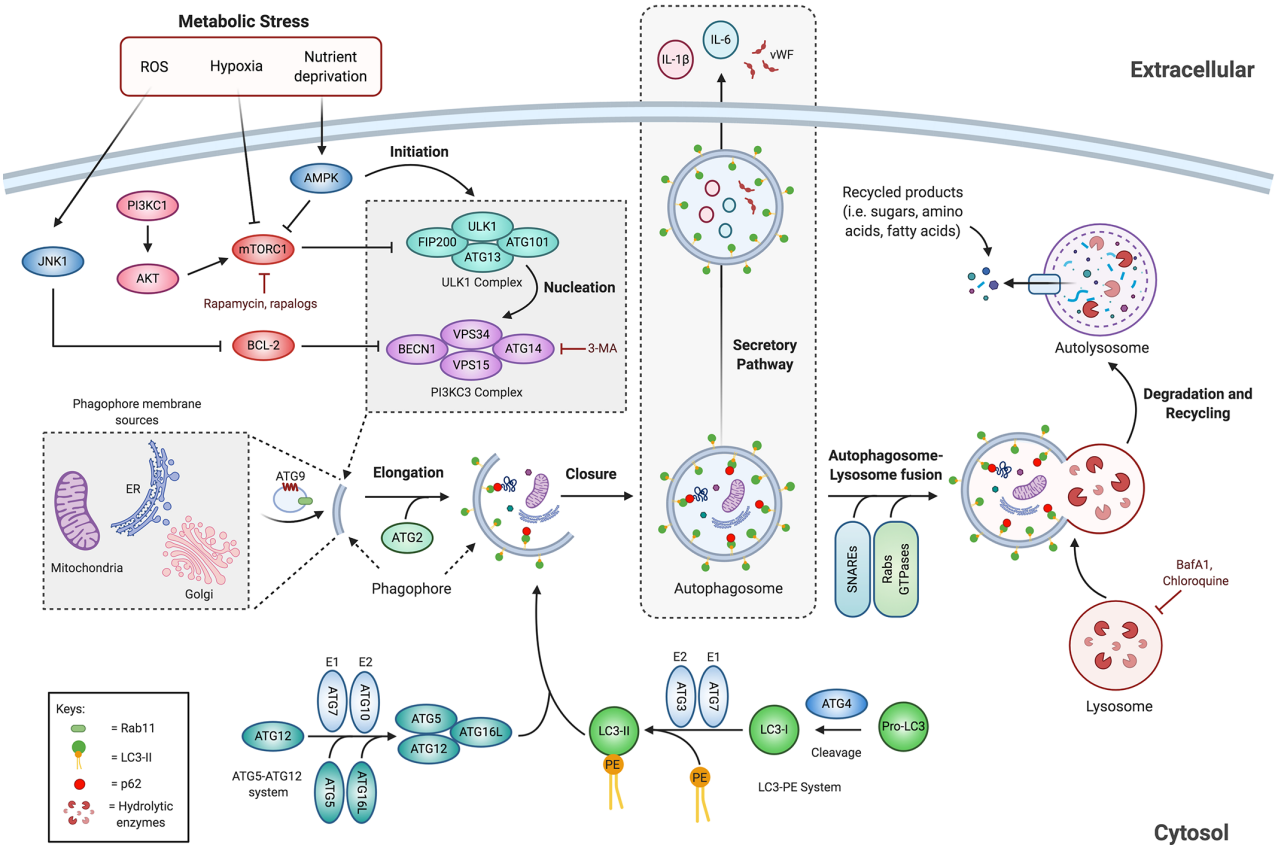


FIGURE 2 | Overview of the autophagic pathway. Autophagy is induced by different stress stimuli including nutritional status, hypoxia and ROS. The autophagic process is initiated by the activity of ULK1 complex, which is regulated negatively by mTORC1 and positively by AMPK. ULK1 complex initiates phagophore nucleation by phosphorylating components of the PI3KC3 complex that leads to the recruitment of several ATGs to assist autophagosome formation. The elongation step involves two ubiquitin-like conjugation complex, the ATG5-ATG12 complex and the LC3-PE complex, which are required for proper phagophore membrane expansion and subsequent closure of the autophagosome. Completed autophagosome fuses with the lysosome to form the autolysosome, where hydrolytic enzymes degrade the enclosed material. The degrading metabolites are transported back to the cytosol for macromolecule synthesis and energy production. In addition, the autophagic machinery is associated with unconventional secretory processes. See the text for additional information. AMPK, AMP-activated protein kinase; ATG, autophagy-related proteins; BafA1, bafilomycin A1; BECN1, Beclin-1; BCL-2, B Cell Lymphoma 2; CQ, chloroquine; ER, endoplasmic reticulum; FIP200, focal adhesion kinase family interacting protein of 200 kDa; JNK1, c-Jun N-terminal kinase 1; LC3, microtubule-associated protein 1A/1B-light chain 3; mTORC1, mammalian target of rapamycin complex 1; PE, phosphatidylethanolamine; PI3K, phosphatidylinositol 3-kinase; SNAREs, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; ULK1, unc-51-like kinase; VPS, vacuolar protein sorting; vWF, von Willebrand factor; 3-MA, 3-methyladenine.

interact with both ubiquitin chains and LC3, and thereby promote ubiquitinated cargo recruitment to autophagosomes for selective degradation (63). As p62 becomes incorporated within the autophagosomes and are degraded in autolysosomes, thus serving as an index of autophagic degradation (60). Upon closure, the autophagosome dissociates from the assembly site and undergoes "maturation" *via* fusion with endosomes and subsequently with lysosomes to form a degradative autolysosome (64, 65). Maturation and autophagosome-lysosome fusion requires several proteins including Rab GTPases, membrane-tethering complexes and soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (66–68).

Finally, the lysosomal hydrolases degrade the autophagic cargo, and the resulting metabolites get recycled and returned to the cytosol through autolysosome efflux transporters, and thus cellular homeostasis is maintained (34, 69, 70).

Autophagy is highly regulated by different signaling pathways implicated in cancer (36, 71, 72). Nutrient starvation is the best-characterized autophagy inductor, where the serine/threonine protein kinase mTOR plays a critical role as an energy sensor (73). Within the human cell, mTOR can be found in at least two distinct multiprotein complexes, referred to as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (74). The mTORC1 complex is considered the primary negative regulator of autophagy (75, 76). Under nutrient-rich conditions, class I PI3K and AKT/PKB activate mTORC1 complex which by phosphorylating ULK1 and ATG13, prevents the induction of autophagy as shown in **Figure 2** (77–80). A sensor of available energy is the AMP-activated protein kinase (AMPK), which is directly activated by a low ATP:ADP ratio (81, 82). Under starving, AMPK directly phosphorylates and inactivates mTORC1 (83). Through

AMPK regulation, the inhibition of mTORC1 and the activation of the ULK complex can initiate the autophagy process (**Figure 2**) (46, 77).

Numerous factors that regulate autophagy are also classified as either oncoproteins or products of tumor suppressor genes [reviewed in (36, 71, 84)]. Thus, autophagy-signaling pathways are caught up in cancer regulation and control (**Figure 2**). Oncoproteins, including the small GTPase RAS, RHEB, and Nuclear Factor-κB (NF-κB), can activate mTORC1 and in consequence inhibit autophagy (85). NF-κB activates autophagy by inducing the expression of proteins involved in autophagosome formation, including BECN1, ATG5, and LC3. Conversely, NF-κB can also inhibit the autophagic process by increasing the expression of autophagy repressors, such like B cell lymphoma 2 (Bcl-2) family members (86). The anti-apoptotic members of the B Cell Lymphoma 2 (Bcl-2)-family bind and sequester BECN1 to prevent autophagy induction (87). On the contrary, tumor suppressors such as the transcription factor Forkhead box O1 (FOXO1) and nuclear p53 are known to induce autophagy (88). Interestingly, ROS production, a hallmark of cancer, and the subsequent activation of the oncogene c-Jun N-terminal kinase1 (JNK1) (89) can lead to the phosphorylation of Bcl-2; this prevents the interaction of this latter protein with BECN1 and thereby induces autophagy (88).

Pharmacological agents are frequently used to either enhance or suppress autophagy (**Figure 2**) (90). A frequent used approach for autophagy induction is mTOR inhibition by rapamycin (91). Conversely, 3-methyladenine (3-MA) can suppress the nucleation stage by inhibiting the PI3K complex, thereby inhibiting autophagosome formation (92). Autophagy can be blocked at later stages resulting in the inhibition of autophagic flux. This refers to the entire process from autophagosome synthesis to lysosomal degradation (93). Bafilomycin A1 (BafA1) is a potent V-ATPase inhibitor that impairs lysosomal acidification and thus the degradation of autophagic cargo (94). By a similar approach, chloroquine (CQ) can inhibit autophagy by increasing the lysosomal pH and therefore reducing the activity of degradative enzymes (95). Accordingly, BafA1 and CQ are commonly used to decrease the autophagic flux.

Although canonically characterized as a degradation mechanism, recent evidence has demonstrated a role for the autophagic machinery in extracellular secretion, a process termed as "secretory autophagy" or more linguistically precise "ATG gene-dependent secretion" (96–98). Accordingly, canonical autophagy involves the fusion of the autophagosomes with lysosomes for cargo degradation, whereas the secretory pathway bypasses this degradative process to allow unconventional extracellular delivery of cytosolic proteins *via* LC3-positive vesicles (**Figure 2**) (99, 100). Even though the molecular pathways in secretory autophagy are not entirely deciphered, the molecular machinery of the degradative processes is required (99). ATG5 and BCN1, together with other factors participating in canonical autophagy, are also activated as part of the secretory pathway (98, 101). The secretory autophagy pathway plays a key role in the progression of several diseases, including cancer (102, 103). It is involved in the secretion of cytokines such as IL-6, IL-8, and IL-1β, damage response mediators such as High mobility group box 1 protein (HMGB1) or ATP, and granule content such as von Willebrand factor (vWF) (104–107). Particularly, autophagy-dependent secretion of IL-6 has been implicated in tumor cachexia (103) and the metastatic potential of Ras-transformed cancer cells (108). The secretory autophagy pathway is reported to mediate cytokine release from cancer-associated fibroblasts, contributing to the development of head and neck squamous cell carcinoma (102). Thus, within the TME, both the cancer cells and supporting stromal cells rely on autophagy-dependent secretion for malignant progression (98).

Interestingly, it has been observed that decreased autophagosome degradation is associated with increased release of extracellular vesicles (EVs) in human malignant cervical and breast cancer cell lines (109). Extracellular secretion may provide a supplementary pathway to maintain cellular homeostasis when the degradative autophagy pathway is blocked (109). These suggest possible crosstalk between degradative and secretory autophagy to maintain cellular homeostasis and tumor cell survival. Furthermore, following pathological stress, there is a reported release of cytosolic proteins, molecular chaperones, harmful nucleic acids and misfolded proteins into the extracellular space through EVs, and this may contribute to tumor progression and metastasis (110, 111).

There is a complex association between cancer and autophagy. While in late-stage disease autophagy promotes tumor progression by providing nutrients to a rapidly dividing yet under vascularized and undernourished tumor, at early stages autophagy may suppress the bourgeoning tumor by suppressing reactive oxygen species and thus limiting genomic instability and, furthermore, promoting an anti-inflammatory microenvironment (36, 112–115).

Noteworthy is that the participation of the coagulation cascade in cancer progression is not limited only to thrombogenesis and thrombocytosis. The hemostatic system is known to promote tumor growth and metastasis, with thrombin increasing proliferation, migration and angiogenesis in preclinical models (116, 117). Circulating Tumor Cells (CTCs) are a marker of poor prognosis and are associated with increased risk of VTE in cancer patients (6, 118). TF has been identified to promote and be present on cancer stem cells (119, 120). Moreover, cancer and adjacent cell expression of PAR1 was recently postulated as a predictive marker of metastasis (121). The plasmatic concentration of Thrombin–Anti-Thrombin III (TAT) complex, a surrogate marker for activated thrombin used to assess coagulation state, inversely correlates with cancer patient survival (6). Independently of a direct role in the clotting process, the TF-FVIIa-PAR2 signaling was also linked to breast and liver cancer progression (23, 122).

Mouse models of cancer have demonstrated that FXa can increase tumor growth (15, 19, 20). Moreover, FXa increased lung, lymph node, liver, kidney metastasis in a syngeneic melanoma model, while promoting vascular permeability and increased infiltration (15). How PAR activation impinges on the autophagic pathway and how this regulates both physiological and pathophysiological processes is still an area requiring further study. We discuss herein that the coagulation system is not

merely a bystander in cancer metastasis, but instead an integral part of the multifaceted approach taken by the malignant cell to survive, propagate, and ultimately exhaust the body's capacity to function. We speculate that the malignant cell may use common mechanisms to hijack immune cells, ECs, hematopoietic cells, and platelets to integrate with the coagulation system for its own end. In this review, we postulate that a common mechanism present in each of these components may be the hijacking of the autophagy pathway, given the reported correlation between CTCs, hypercoagulable state and reduced survival in cancer. We examine the evidence for shared mechanisms and pathway integration to better understand cancer pathophysiology and to uncover novel druggable targets for the oncology clinic.

AUTOPHAGY REGULATES THROMBUS FORMATION THROUGH MEGAKARYOCYTE DIFFERENTIATION, PLATELET PRODUCTION, AND PLATELET ACTIVATION

Autophagy Is Implicated in Megakaryopoiesis and Thrombopoiesis

The body produces 1×10^{11} platelets per day to maintain platelet count through the processes of megakaryopoiesis and thrombopoiesis (123). As schematically represented in **Figure 3**, within the bone marrow, the process of megakaryopoiesis gives origin to MKs from Hematopoietic Stem Cells (HSCs) through successive lineage commitment steps, followed by a maturation process. HSC differentiate through sequential steps into multipotent progenitors (MPPs), common myeloid progenitors (CMPs), bipotential MK-Erythroid progenitors (MEPs), and unipotent MK progenitors (MKPs), which then mature into MKs (124–127). The principal growth factor regulating steady-state megakaryopoiesis and thrombopoiesis is Thrombopoietin (TPO), which influences almost every step of the differentiation and maturation process. TPO is involved in HSC self-renewal, the proliferation of MKPs, MK maturation, and platelet production. TPO binds to the Thrombopoietin receptor (Mpl) activating JAK2 signaling, STAT3/5 and MAPK pathways and in this way positively regulates MK and platelet production (128).

Autophagy and megakaryocytic differentiation are overlapping processes. An up-regulation of BECN1 and LC3II precedes megakaryocytic differentiation. Moreover, autophagy is necessary for MK differentiation *in vitro* (129). Human CD34+ hematopoietic progenitor cell differentiation into MKs is slowed down by the autophagy inductor rapamycin, resulting in a lower percentage of and smaller MKs (130). It was further shown that ATG2B and the GSK3B-interacting protein (GSKIP) enhanced the differentiation of CD34+ progenitors and MKPs into MKs by increasing progenitor TPO sensitivity (131).

Moreover, it seems that autophagic flux regulation and its counterbalance of the apoptosis pathway is a crucial aspect during megakaryopoiesis (132). Interestingly, TPO stimulation results in TPO-Receptor internalization and targeting to the autolysosome for degradation (133, 134). Besides the canonical ER-Golgi route, the receptor traffics to the membrane through secretory autophagy (133, 134). Based on these studies, steady-state megakaryopoiesis and thrombopoiesis may be maintained by MAPK activation, which in turns targets the TPO receptor to autolysosomes and thus decreases TPO sensitivity in MPPs. However, an increase in p62 and thus disruption of the autophagic flux is observed (129), indicating that TPO could increase autolysosome formation but not necessarily degradation, thus favoring secretory autophagy. TPO activation of MAPK signaling may be promoting autophagy and therefore reducing TPO sensitivity; however, sensitivity could increase through secretory autophagy mediated by BECN1, ATG2B, and GSKIP (**Figure 3**). Therefore, the balance between degradative and secretory autophagy may play a key role in TPO sensitivity and steady-state megakaryopoiesis.

Further demonstrating the dependence of the autophagic pathway in steady-state megakaryopoiesis, VPS34, a protein required at the "nucleation stage", is implicated in MK migration, Demarcation Membrane System (DMS) development, pro-platelet formation and platelet release (135, 134). Although rapamycin strongly inhibits polyploidization and pro-platelet formation (130, 137), hematopoietic lineage-specific *Atg7* deletion demonstrated that the autophagy machinery is necessary for thrombopoiesis (138). *Atg7* deletion not only impaired autophagy but also megakaryopoiesis, MK differentiation and thrombopoiesis. In particular, *Atg7* knockout (KO) mice presented fewer platelets and failed to maintain hemostasis (138). In contrast, a separate study using $Atg7^{f/f}$; PF4-Cre mice failed to demonstrate a role for *Atg7* in thrombopoiesis (139). Differing results may be explained by HSC relying exclusively on ATG7-dependent autophagy; however, differentiated cells may trigger compensatory non-canonical signaling pathways (140). Given the role of *Atg7* in the initiation of canonical autophagy, this process appears to be crucial for both MK and platelet production and function.

Under stress conditions such as injury and infections, platelets are rapidly consumed, representing a high risk for health. Inflammatory signaling leads to a 10-fold increase in platelet production and platelet size (141). As shown in **Figure 3**, the regulatory mechanism of thrombopoiesis is mainly attributed to IL-6 promoting TPO secretion from the liver (142). However, inflammatory signaling could have a more direct effect as STAT3 activation is required for MKP expansion, MK maturation, and platelet production in a TPO independent fashion (143). Further mechanisms may involve the potentiation of megakaryopoiesis through TLR-mediated activation of quiescent stem-like MK-committed progenitors and IL1a driven MK rapid cytoplasmic fragmentation (144, 145). While autophagy regulation by inflammatory stimuli is well studied (42, 97), to our knowledge, there are no reports to date implicating autophagy in inflammation-mediated thrombopoiesis.

Taken together, published data strongly suggest the relevance of the autophagic process during steady-state megakaryopoiesis. Interestingly, autophagic activity may vary between stages of commitment and differentiation and may be involved in TPO

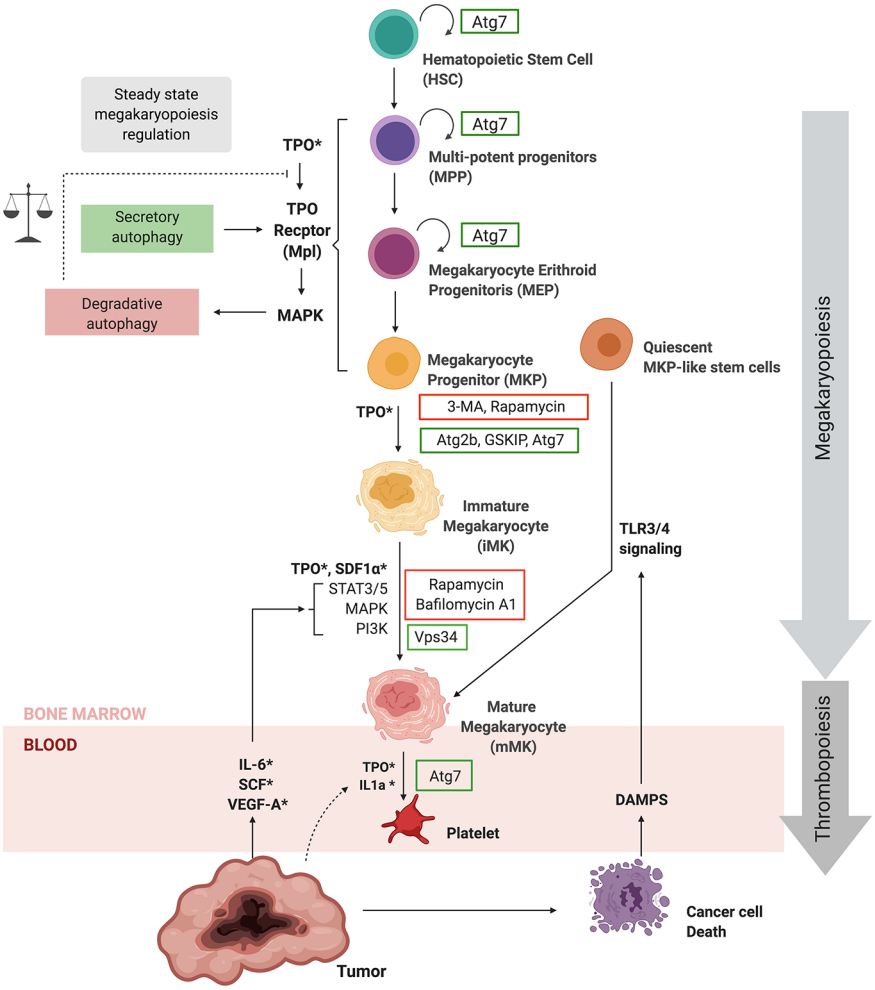


FIGURE 3 | Autophagy regulates megakaryopoiesis and thrombopoiesis. Hematopoietic stem cells give origin to megakaryocytes (MKs) through megakaryopoiesis by a succession lineage commitment steps, followed by a maturation process. TPO is the main growth factor regulating steady-state megakaryopoiesis and thrombopoiesis, which bind to the Thrombopoietin receptor (Mpl). Autophagic regulation of the Thrombopoietin receptor plays a key role in steady-state megakaryopoiesis. ATG7, ATG2B, and GSKIB are necessary for megakaryopoiesis, while BafA1, CQ and rapamycin have an impact at different steps of the differentiation process. Furthermore VPS34 is also necessary for MK maturation. Inflammatory signaling through TLR/NFkB pathways promotes the differentiation of quiescent MKP–like stem cells into megakaryocytes during emergency megakaryopoiesis and thrombopoiesis upon injury. Importantly, cancer increases systemic levels of TPO, IL-1α, IL-6, SCF, VEGF-A, and DAMPs that could not only enhance steady state thrombopoiesis but also emergency megakaryopoiesis and thrombopoiesis. The regulation of autophagy during megakaryopoiesis and thrombopoiesis still requires further investigation. For more information please refer to text. ATG, Autophagy-Related Gene; BafA1, Bafilomycin A1; BECN1, Beclin-1; CQ, Chloroquine; DAMPs, Damage-Associated Molecular Patterns; IL-1α, Interleukin 1 alfa; IL-6, Interleukin 6; MAPK, Mitogen-Activated Protein Kinase; PI3K, Phosphatidylinositol 3-Kinase; STAT, Signal transducer and activator of transcription 3; Thrombopoietin, TPO; VPS, Vacuolar Protein Sorting; 3-MA, 3-methyladenine; SDF-1, stromal cell-derived factor 1.

sensitivity and pro-platelet production in steady-state thrombopoiesis. The relationship between autophagy and inflammatory signaling, as well as the existence of inflammatory induced thrombopoiesis, raises the question if these processes are connected. Further studies are required to clarify the role of autophagy during megakaryopoiesis and thrombopoiesis.

Crosstalk Between Coagulation Cascade and Autophagy During Platelet Activation

Despite being a developing field of research, the machinery and cellular structures of autophagy have been identified to be present and operative in platelets, indicating that platelets undergo basal autophagy much like nucleated cells. Autophagosome-like structures in platelets were first described during the 70s (146) and recently confirmed by super-resolution microscopy (147). Furthermore, the presence of autophagy-related structures and proteins are also evidenced in resting platelets (139, 147–150). Starvation or rapamycin treatment increases the autophagy in platelets in an ATG5-dependent fashion, which is reversed by 3-MA (151, 152). Although still under study, the physiological relevance of autophagy in platelets appears to be related to their activation. Firstly, autophagy can be

induced during platelet activation by hemostatic agonists (139, 151). Secondly, defective platelet activation has been reported in *Atg7*-, *Atg5*-, *Becn1*-, *Vps34*- knockout mice (135, 139, 153).

Platelet activation occurs upon collagen binding to platelet glycoproteins (such as GPVI), thrombin engagement of PARs, and ADP activation of P2X receptors [reviewed in (154)]. Activation of these pathways triggers positive feedback, with platelets releasing their granules containing more agonists such as prothrombin, ADP and among other growth factors (**Figure 4**). In parallel, integrins are upregulated on platelet membranes, allowing platelet aggregation and ultimately clot formation (155). Noteworthy, is that *Becn1* KO and platelet specific *Vps34* KO mice display defective collagen-induced platelet aggregation, adhesion and thrombus formation (136, 152, 153). This association still needs further investigation as *Becn1* is also dampened in ECs, among other cells relevant for hemostasis.

Interestingly, platelet specific *Vps34* KO showed impaired thrombus formation in two independent studies, indicating that

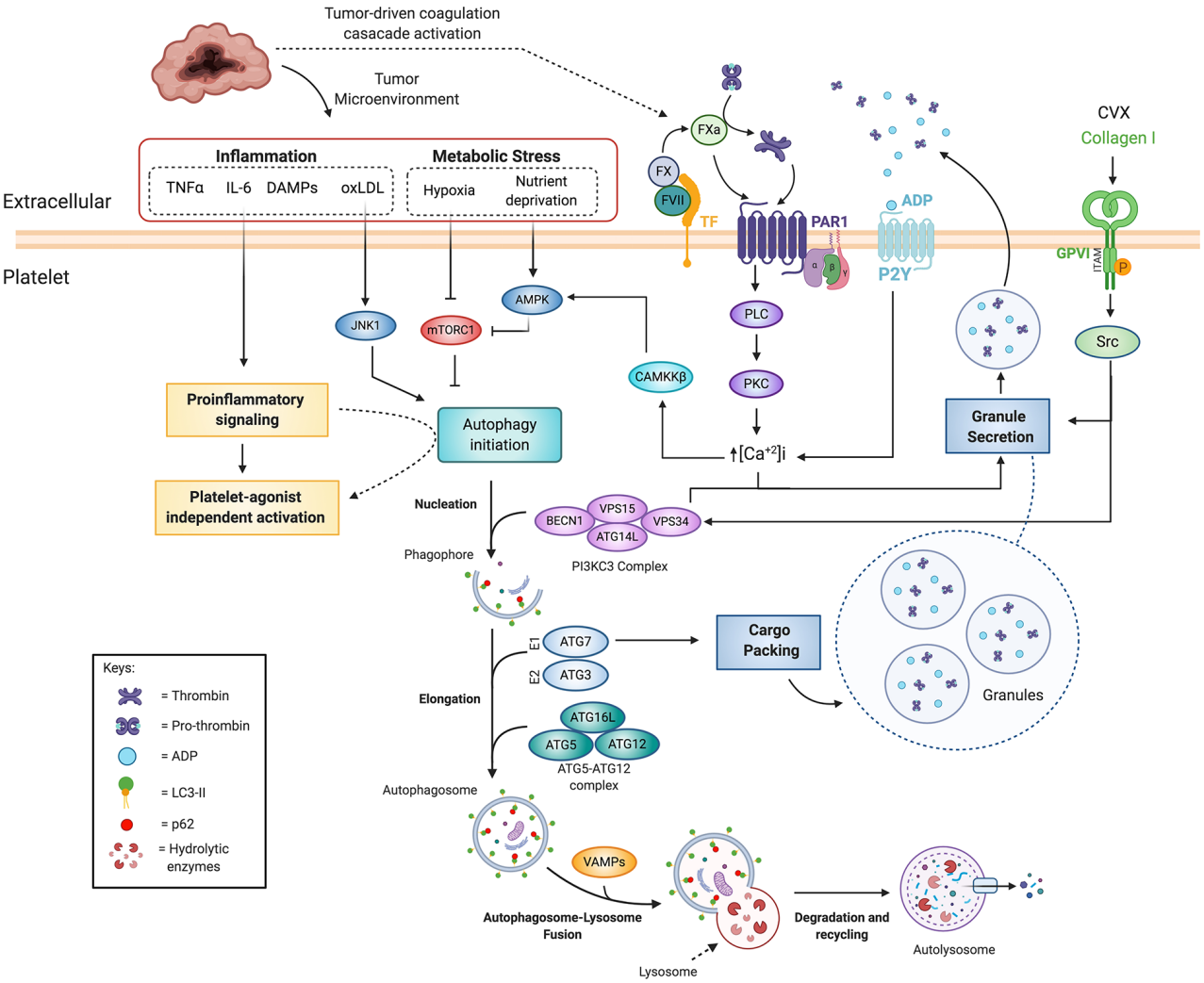


FIGURE 4 | Coagulation cascade and autophagy crosstalk in platelet activation and cancer-associated thrombosis. Platelet agonists such as thrombin, ADP and collagen can increase autophagic flux, which is necessary for efficient platelet activation. PAR activation and P2Y engagement activate the PLC-PKC-Ca^{2+} signaling, which has been shown to increase platelet autophagic flux during platelet activation, possibly through CAMKKB. Additionally, autophagy proteins such as BCN1, VPS34, ATG7, ATG5, and VAMPs have been shown to be necessary for platelet activation, cargo packing and granule secretion. Tumors increase systemic inflammation and locally generate both hypoxia and nutrient deprivation, which cannot only promote platelet activation but also autophagy through JNK and AMPK activation, and mTORC1 inhibition. Pro-inflammatory signaling through increased levels of extracellular TNFα, IL-6, and IL-8, which are frequently elevated in cancer patient serum samples, is involved in both platelet activation and possibly modulating platelet autophagy. ADP, adenosine diphosphate; AMPK, AMP-activated protein kinase; ATG, autophagy-related proteins; BECN1, Beclin-1; CAMKKB, calcium/calmodulin-dependent protein kinase kinase 2; CVX, convulxin; DAMPs, Damage-Associated Molecular Patterns; FVII, Factor VII; FX, Factor X; FXa, activated FX; GPVI, Glycoprotein VI; IL-6, Interleukin 6; IL-8, Interleukin 8; JNK1, c-Jun N-terminal kinase 1; LC3, microtubule-associated protein 1A/1B-light chain 3; mTORC1, mammalian target of rapamycin complex 1; oxLDL, Oxidized low-density lipoprotein; PAR, protease activated receptor; PE, phosphatidylethanolamine; PI3K, phosphatidylinositol 3-kinase; PKC, Protein kinase C; PLC, Phospholipase C; TF,Tissue Factor; TNFα, Tumor Necrosis Factor α; VPS, vacuolar protein sorting; VAMP, Vesicle associated membrane proteins.

platelet VPS34 is necessary for the clotting process (133 153). Accordingly, thrombin or C Reactive Protein (CRP) treatment significantly increases VPS34 dependent PI3P production in platelets while in *Vps34*$^{-/-}$ platelets and human platelets treated with 3-MA displayed impaired responses to thrombin, collagen and ADP (133). Furthermore, *Vps34*$^{-/-}$ platelets displayed impaired aggregation, dense granule secretion and decreased levels of Syk and PLCγ2 phosphorylation (153). In line with these findings, 3-MA impaired human platelet activation in response to Convulxin (a GPVI agonist) and thrombin, suggesting involvement of VPS34 in platelet activation downstream of PARs and GPVI (153). Although *Vps34*$^{-/-}$ platelets showed reduced mTOR signaling and increased LC3-II levels, the authors of this study did not demonstrate an association between autophagy and platelet activation (153). Alternatively, they proposed that VPS34 promotes PI3P–guided NADPH oxidase assembly and subsequent ROS generation, supporting PAR- and GPVI-mediated platelet activation (153). However, a separate study showed an increased rate of secretion in response to platelet agonists in VPS34-deficient platelets *ex vivo* and underflow conditions (133). Moreover, under arterial flow, VPS34-deficient platelets display an inefficiency in recruiting circulating WT platelets to the growing thrombus (133). These authors proposed that VPS34 production of PI3P contributes to the spatiotemporal regulation of granule secretion, possibly by recruitment of intracellular proteins that regulate granule fusion and secretion (133). Interestingly, ROS production in platelets suppresses the downstream activity of the PI3K/AKT/mTOR signaling pathway promoting autophagy and consequently exacerbating platelet aggregation (156). These observations raise the possibility that VPS34 mediation of ROS production may promote autophagy and subsequently platelet aggregation; however, this hypothesis still needs to be tested.

Interestingly, 65% of mice with a platelet specific deletion of *Atg7* (*Atg7*$^{f/f}$;*PF4-Cre*/$^{+}$) were unable to arrest bleeding 10 min after tail transection despite no significant effects on platelet counts or volumes (139), further demonstrating that selective autophagy is an aspect for platelet activation. In the same line, it has been proposed that *Atg5* enables mitophagy in platelets, which is a requirement for correct platelet activation (157).

The concept that autophagy could be promoted downstream of platelet agonist signaling, and that in turns regulates platelet activation, is supported by the observation that autophagy inhibitors block platelet aggregation and adhesion. Pre-incubation of platelets with 3-MA, BafA1 or CQ is shown to inhibit Collagen I (COL1)- and thrombin-stimulated aggregation (151). Interestingly, thrombin reduces LC3II levels, indicating an increase of the autophagic flux upon platelet activation (139). Similarly, a PAR1 peptide and ADP both mediated a reduction in LC3II (139). LC3II reduction requires degradation through canonical autophagy which is mediated by key downstream elements of platelet activation signaling cascade, including phospholipase C, protein kinase C, Ca^{2+}, and Src-family kinases, as shown in **Figure 4** (139) Although Src-family kinases seem to be upstream of LC3II increase in platelets, the specific mechanism has yet to be elucidated. On the other hand, extrapolating mechanisms reported in other cell types, Ca^{2+} may promote autophagy through CAMKKβ/AMPK/mTOR signaling (158). Hence, GPCR mediated platelet activation and subsequent increase in $[Ca^{2+}]_i$ could promote autophagy through this pathway (**Figure 4**). However, AMPK-independent Ca^{2+} regulation of autophagy has also been described (159). Thus, the possibility exists that Ca^{2+} promotes autophagy through a different mechanism.

The evidence of autophagy in resting platelets and, more importantly, of an increased autophagic flux regulation during platelet activation, clearly points to crosstalk between platelet agonists and autophagy and strongly suggests that autophagy is essential for coagulation. Furthermore, deficiency in key mediators of autophagosome formation such as VPS34 and ATG7 are associated with secretion and packaging of platelet granules, a fundamental aspect in thrombus formation. Taken together, platelet agonists signaling through PARs and GPVI could activate the autophagy signaling machinery, potentially mediated by Ca^{2+} and Src signaling, to promote autophagosome formation, which in turn promotes efficient granule packing and secretion (**Figure 4**).

Autophagy Implication in Cancer-Associated Thrombocytosis Through the Regulation of Platelet Production and Activation

It is established that thrombocytosis at diagnosis correlates with enhanced tumor invasion, metastasis, and poor prognosis in several solid cancers. Mean platelet volume was recently proposed as a diagnostic biomarker for lung cancer (160), while platelet counts have been correlated with stage and survival in melanoma patients, were thrombocytosis at diagnosis was significantly associated with distant metastasis (161). The current line of thought is that tumors benefit from thrombocytosis through platelet interaction with the CTCs, the latter encapsulating the tumor cells protecting them from NK cells and promoting the maintenance of an epithelial to mesenchymal transition (EMT) state (119, 162–165).

Altered platelet production in cancer could be explained by inflammation-mediated thrombopoiesis. A hypothesis is that tumor production of IL-6 promotes megakaryopoiesis *via* hepatic TPO, leading to thrombocytosis (142, 166). However, other plasmatic thrombopoietic cytokines such as Stem Cell Factor (SCF), Interleukin 1 alpha (IL-1α), Tumor Necrosis Factor alpha (TNF-α), stromal cell-derived factor 1 (SDF1), and Vascular Endothelial Growth Factor A (VEGF-A) are increased in cancer patients, providing alternative pathways for thrombopoiesis that could be independent or complementary to the IL-6/TPO axis. The SDF1a-CXCR4 axis is independent of the TPO/Mpl axis in murine models (167). Moreover, VEGFR1 promotes MKP maturation, possibly through CCXR4 up-regulation, leading to increased platelet counts *in vivo* (168, 169). Likewise, VEGFR2 activation increases MK proliferation, survival and differentiation (170).

Moreover, it has been reported that quiescent MKPs can rapidly differentiate into mature MKs and replenish platelet counts in response to inflammatory stimuli (144). This may be triggered by malignant signaling, as Toll-Like Receptor (TLR) activation is implicated in both hematological and solid tumors (171). As previously discussed, autophagy inhibition in early megakaryopoiesis appears to impair MK maturation, reduce platelet formation, and affect platelet function. Although many of the thrombopoietic cytokines such as TPO, IL-6, TNF-α, and VEGF have been associated with autophagy in MK or other cell types (131, 172) it remains unknown if, through deregulation in cancer, autophagy is central to promoting platelet production and thus sustaining a vicious cycle between malignancy and thrombopoiesis.

Suggesting that platelet autophagy is somehow deregulated in cancer, Lewis et al. described an increase in autophagosome-like structures in platelets from cancer patients (146). In malignancy, an obvious path for platelet autophagy regulation and platelet activation is the deregulation of the coagulation-signaling cascade. As previously discussed, platelet agonists such as thrombin, ADP and collagen can increase autophagic flux which is necessary for efficient platelet activation. Pro-inflammatory cytokines such as TNFα, IL-6, and IL-8, which are frequently elevated in cancer patient serum samples (173), are also involved in both platelet activation and autophagy. TNFα was shown to promote the platelet activation independently of platelet agonists, increasing TF expression, thrombin generation and subsequent clot formation (174). Other pro-inflammatory cytokines such as IL-6 and IL-8 and NF-κ B activation can also promote platelet activation (175, 176). TLR, TNFα, NF-κB, and JAK/STAT signaling pathways have been widely reported to regulate autophagy (42, 172, 177); however, this has yet to be evaluated in platelets.

ROS regulates autophagy in various cells by modulating the PI3K/AKT/mTOR pathway, and recent studies show that this is also true in platelets (156). High blood levels of oxidized LDL (oxLDL) have been reported in cancer patients and associated with metastasis in breast, ovarian, gastric and prostate cancer (178–180). Noteworthy, these cancers are also associated with a higher risk for thrombosis (181). ox-LDL can increase platelet activation in a VPS34 dependent manner, suggesting the involvement of the autophagy process (156). In line with these findings, the Oxidized low-density lipoprotein receptor 1 (LOX-1), recognizes and binds activated platelets assisting in the formation of a thrombus (182).

Furthermore, a separate study revealed that autophagy was activated in platelets through an oxidative stress-induced JNK pathway, which was evidenced by increased co-localization of LC3II with the Lysosomal Associated Membrane Protein 1 (LAMP1), suggesting enhanced autolysosome formation, after hydrogen peroxide treatment (152). It was also observed that mitophagy reduced phosphorylated p53, thus preventing apoptosis, and conversely the absence of mitophagy resulted in increased thrombosis (152). In this way, tumor micro-environmental cues such as ROS could alter platelet selective autophagy of mitochondria and thereby regulate platelet activation and thrombus formation.

Taken together, tumor-derived soluble factors could promote thrombopoiesis and potentially deregulate autophagy in hematopoietic progenitors and MKs. TME factors may regulate autophagy and subsequent platelet activation, furthermore, a high coagulative state and platelet-derived factors may promote tumor growth, metastasis and chemotherapy resistance.

COAGULATION AND AUTOPHAGY CASCADE IN CANCER CELLS

TF and its protease products, such as Thrombin and FXa, are associated with cancer progression and cancer-related thrombosis (15, 20, 119, 183). Furthermore, this association may not be solely related to TF expression in the primary tumors as results have now shown that circulating microparticles carrying TF are 16- to 26-fold higher in pancreatic cancer patients with thrombosis when compared to healthy controls (183). There is emerging evidence that there is an involvement of the autophagy cascade in the mechanism controlling and promoting cancer progression and cancer-related thrombosis (23, 184). Proteins of the autophagy pathway are associated with cancer cell chemotherapy and radiation sensitivity through the alleviation of cellular stress and the dampening of apoptosis. Somewhat paradoxically, BECN1 has been postulated as a tumor suppressor due to its role in regulating p53 stability (185). As already mentioned, mTORC1 is the master negative regulator of the autophagic pathway and plays a critical role in cancer cell growth and progression (186, 187). Due to insufficient vascularization, tumors experience nutrient deprivation and hypoxia (188–190). In this scenario, mTORC1 may act as a restriction point between proliferation and differentiation (191). A hypoxic microenvironment also leads to an increase in both TF and FVII, correlating with tumor progression, local invasion, distant metastasis and therapeutic resistance (192).

As shown in **Figure 5**, thrombin mediated-PAR1 activation signals through PI3K/AKT/mTORC1 and activates Hypoxia-Inducible Factor-1 alpha (HIF-1α) (193), a well-known mediator of tumor survival, EMT, angiogenesis, and metastasis (193–195). Activation of PAR2 also leads to the downstream activation of the PI3K/AKT signaling pathway, promoting cell migration and invasion in both oral squamous and renal cell carcinoma (196, 197). In accordance, activation of both PI3K-AKT and mTORC1 signaling pathway by TF-FVIIa-Xa complex mediated by PAR1 and PAR2 has been associated with enhanced cell migration in a human breast cancer cell line (198, 199). Furthermore, recombinant TF, recombinant FVII or a PAR2 agonist upregulated mTORC1 signaling pathway in a hepatocellular carcinoma cell line (23). Moreover, levels of the autophagic marker LC3-II and the coagulation proteins TF, FVII and PAR2 were inversely correlated in human hepatocellular carcinoma tissues (23), suggesting a possible role of the coagulation pathway on autophagy suppression in cancer. In further accordance with an interaction between PAR signaling and autophagy, it was reported in human kidney tubular

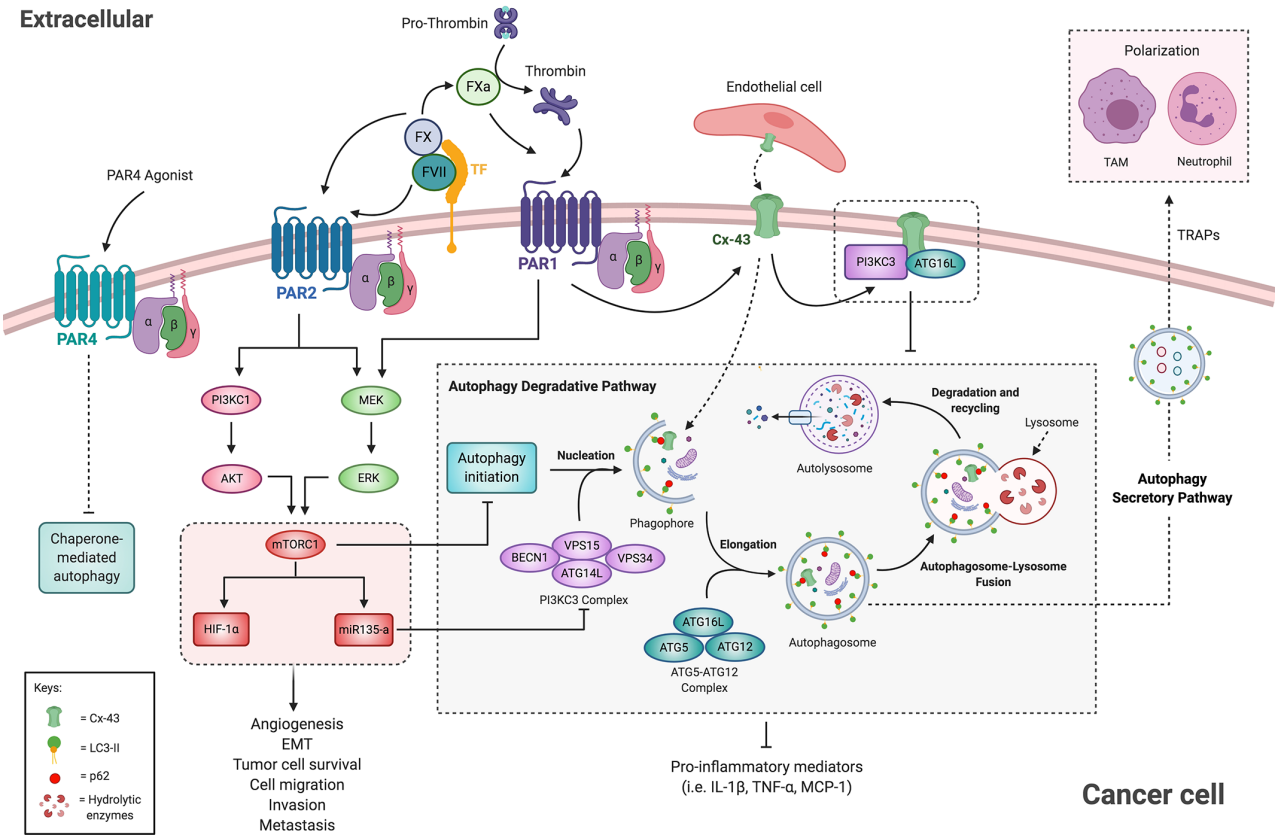


FIGURE 5 | Crosstalk of the coagulation cascade and autophagy pathway in cancer cells. The activation of PAR1 and PAR2 by coagulation factors suppresses the autophagic pathway in an mTORC1-dependent manner. mTORC1 upregulates HIF1-α and miR135, both implicated in cancer and in autophagy regulation. PAR1 activation leads to Cx-43 up-regulation, which may impair autophagy by sequestering ATG16L and the PI3KC3 complex to the membrane. In turn, the autophagic pathway mediates PAR1 degradation. Additionally, PAR4 selective stimulation downregulates proteins associated with chaperone-mediated autophagy. Therefore, autophagy suppression by PARs activation leads to increased pro-inflammatory microenvironment and enhanced cancer progression and metastasis. Moreover, the autophagy secretory pathway participates in TRAPs release, which suppress anti-tumor immune response and thereby facilitate tumor progression. See the text for additional information. Cx-43, Connexin 43; EMT, Epithelial to Mesenchymal Transition; ERK, Extracellular-signal Regulated Kinase; FVII, Factor VII; FX, Factor X; FXa, activated FX; HIF1-α, Hypoxia-Inducible Factor-1; LC3, microtubule-associated protein 1A/1B-light chain 3; MEK, mitogen-activated protein kinase; MCP1, monocyte chemoattractant protein 1; Monocyte mTORC1, mammalian target of rapamycin complex 1; PAR, protease-activated receptor; PI3K, phosphatidylinositol 3-kinase; TAM, tumor-associated macrophage; TF, tissue factor; TRAPs, tumor cell-released autophagosomes.

epithelial cells that PAR2 acting through the PI3K/AKT/mTOR pathway suppressed the process of autophagy, by affecting ATG5 and ATG12, and thus decreasing autophagosome formation (24). Additionally, downregulation of autophagy is associated with enhanced secretion of inflammatory mediators, such as IL-1β, TNF-α, and Monocyte Chemoattractant Protein-1 (MCP-1) (24). The aforementioned data suggest that activation of PAR2 by TF-FVII-Xa suppresses the autophagic pathway in a PI3K/AKT/mTOR-dependent manner (184), contributing to the generation of an inflammatory microenvironment that may lead to increased cancer cell migration and invasiveness. In this way, PAR signaling could directly promote tumor growth.

Interestingly, allograft models indicate that PAR1 promotes tumor growth by mediating immune escape. Tumor depletion of TF or PAR1 in allograft studies showed that $CD8^+$ T cells effectively eliminated *Par1* KO cancer cells in immune-competent mice (21). Similarly, in a separate study, cancer cell derived-extracellular vesicles and tumor-released autophagosomes (TRAPs) mediated immune escape by T cell suppression. In particular, these vesicles induced autophagy and activation and polarization of neutrophils and macrophages into an anti-inflammatory phenotype that promoted tumor growth and immune escape in a Programmed Death-ligand 1 (PDL-1) dependent fashion (200, 201). Furthermore, blockade of autophagy in tumor cells promoted the switch of macrophages into the anti-tumor M1-like phenotype and restored immune function of tumor-infiltrating lymphocytes (TILs) (201). A tempting hypothesis would be that PAR signaling mediates the secretion of TRAPs through autophagy regulation, thus promoting secretory autophagy while decreasing degradative autophagy, which may promote both cancer cell survival and immune escape.

While extracellular vesicles may allow intercellular communications throughout the body, direct communication between tumor cells and ECs mediated by connexins may also be critical to tumor cell extravasation at a potential metastatic site (202). Connexin 43 (Cx-43) has been reported as an obstacle and a promoter of cancer progression. As examples, Cx-43 may obtain tumor suppressor gene status as its loss contributes to metastasis (203–205). Conversely, expression of Cx-43 has also been shown to enhance tumor metastasis through increased attachment and communication with the vascular endothelium (**Figure 5**) (206–209). In metastatic melanoma cell lines, PAR1 silencing decreased Cx-43 expression as well as cancer cell attachment to ECs and extravasation, suggesting that PAR1 contributes to invasion and metastasis *via* regulation of Cx-43 (210). However, it should be noted that most connexins are characterized by a rapid turn-over mediated by different degradation pathways, including autophagy, where p62 served as a cargo-recognition factor, forming a bridge between ubiquitinated Cx-43 and LC3, thereby leading it's to degradation (211–213). In turn, connexins might negatively regulate the autophagic process at initial stages (214). As represented schematically in **Figure 5**, Cx-43 may recruit ATG16 and the PI3K-complex to the plasma membrane, limiting their availability and capacity for regulating autophagy (212). Thus, Cx-43 up-regulation induced by PAR1 could impair autophagy leading to enhanced migration and invasion in cancer.

Furthermore, PAR4 is also associated with cancer development (215), and its activation in esophageal squamous cell carcinoma (ESCC) leads to the downregulation of proteins associated with chaperone-mediated autophagy, such as the Heat shock protein family A (Hsp70) (216). This evidence strongly suggests that PARs activation mediates cancer progression by regulating signaling pathways associated with the autophagy process (**Figure 5**).

MicroRNAs (miRNAs) are now widely reported to play critical roles in the modulation of autophagy in cancer cells and as potential markers for cancer detection (217, 218). It is thus unsurprising that an association exists between coagulation-mediated autophagic suppression and tumor malignancies involving miRNAs participation (25). Huang and collaborators observed in hepatocellular carcinoma tissues that elevated levels of both miR-135a and FVII were associated with tumor stage, recurrence, microvascular invasion, and decreased disease-free survival (**Figure 5**) (25). Moreover, in Hep3B cells treated with recombinant TF, FVIIa, or a PAR2 peptide agonist, the expression levels of miR135a were increased. They further demonstrated that expression of this miRNA was dependent on mTOR levels and that miR-135a acts as a downstream effector of PAR2 activation, abrogating the autophagic process in an mTOR dependent manner (25). In the same line, in breast cancer cells, miR-142-3p inhibits autophagy by targeting HMGB1 (219), which can be delivered by platelets and is associated to autophagy in other cell types (220). Platelet-derived exosomes contain miR-126 (221), which inhibition enhances autophagy as determined by LC3-II increased and p62 decreased protein levels in ESCC (222). These few examples demonstrate that miRNAs secreted by the coagulation through the release of platelet exosomes may impinge on autophagy-related pathways and mediate cancer cell progression. Thus, future investigation should seek to evaluate the interplay between coagulation, autophagy and microRNA in the same biological models.

COAGULATION CASCADE AND AUTOPHAGY IN THE TUMOR MICROENVIRONMENT

The tumor microenvironment (TME) plays a principal role during tumor growth and metastasis. Within the tumor, ECs, immune cells and stromal cells alter their phenotype into a tumor-promoting state through secretion of growth factors and cytokines that sustain cancer cell survival and proliferation, while dampening the immune response. Interestingly, all these cells are subjected to stressors generated by cancer cells, such as glucose deprivation, hypoxia and inflammatory signaling; thus, autophagy plays a central role in the regulation of the TME. The TME is also a source of pro-thrombotic proteins, such as TF and FX, and thus in the subsequent section we will discuss how the interplay between the coagulation and autophagy cascades could maintain and promote tumor growth.

Coagulation Cascade and Autophagy in the Tumor Endothelium

During metastasis, cancer cells acquire an invasive phenotype and detach from the primary tumor and enter the bloodstream in a process called intravasation. Subsequently, cancer cells migrate out of the bloodstream by the process of extravasation and establish metastatic foci at distant organs (223). Fundamental to both these processes is endothelial permeability, which is promoted by the coagulation factors thrombin and FXa (15, 224). Interestingly, recent studies have shown that autophagy is involved in thrombin-induced endothelial dysfunction. Thrombin promotes VE-cadherin disassembly and degradation, allowing endothelial hyper-permeability in through BECN1 (225). Likewise, *in vitro* knockdown of *Atg5* inhibited thrombin-induced actin stress fiber formation and VE-cadherin loss at the cell surface, thus preventing endothelial barrier dysfunction (226). Furthermore, pharmacological inhibition of autophagy with 3-MA, BafA1, or CQ can abrogate thrombin-induced hyperpermeability (227). Thus, in response to thrombin, VE-cadherin degradation through autophagy may lead to vessel hyperpermeability, as shown in **Figure 6**.

Poor vascular integrity contributes to the TME. Thrombin induces macrophage migration Inhibitory Factor (MIF) secretion from ECs, and the process of autophagy is involved in MIF-mediated endothelial hyperpermeability. Chao and colleagues showed that blocking autophagy attenuated thrombin-induced hyperpermeability in EC lines. Furthermore, blocking of autophagy or MIF effectively alleviated vascular leakage (227). These data suggest that endothelial permeability

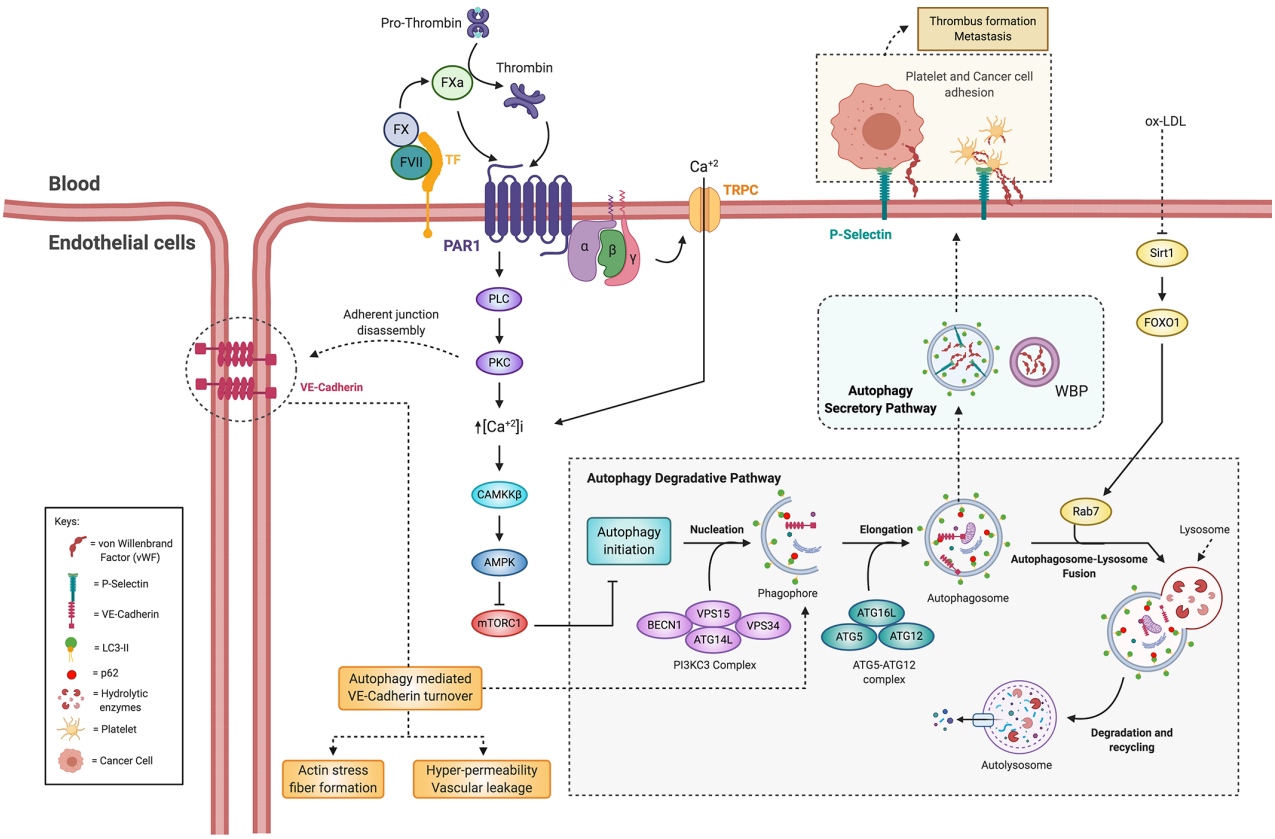


FIGURE 6 | Crosstalk between the coagulation cascade and autophagy on tumor-associated endothelial cells. Thrombin-induced endothelial hyperpermeability and endothelial secretion of thrombosis-promoting factors are dependent on autophagy. Activation of the coagulation cascade and PAR1 induces an increase in intracellular Ca^{+2}, which allows activation of AMPK by CaMKKB. Thus, the activation of AMPK induced by PARs could induce autophagy. Disassembly and degradation of VE-cadherin and the formation of actin stress fibers promoted by thrombin through autophagy could allow endothelial hyperpermeability. The secretion of vWF and P-selectin is mediated by the secretory pathway autophagy. Autophagosomes contain vWF and allow its release from endothelial cells. The secretion of vWF and P-selectin stimulated by ox-LDL is mediated by the inhibition of Sirt1/FoxO1 signaling by preventing fusion of autophagosome with lysosome. These factors allow adhesion of cancer cells and platelets to the endothelium, thus promoting cancer-related thrombosis and metastasis. FVII, factor VII; FX, factor X; FXa, factor X activated; TF, tissue factor; PAR1, protease-activated receptor 1; TRPC, transient receptor potential canonical; PLC, phospholipase C; PKC, protein kinase C; CaMKKB, calcium/calmodulin-dependent protein kinase kinase B; AMPK, AMP-activated protein kinase; mTORC1, mammalian target of rapamycin complex 1; VE-cadherin, vascular endothelial cadherin; vWF, von Willebrand factor; WBP, Weibel-Palade bodies; Sirt1, sirtuin 1; FoxO1, forkhead box protein O1; ox-LDL, oxidized low-density lipoprotein.

modulated by coagulation factors is dependent on autophagy (**Figure 6**), although further studies are required to evaluate these processes in the context of cancer.

How coagulation factor signaling pathways interact with autophagy is still an open question. However, upon ECs we know that thrombin binding to PAR1 initially induces Ca^{+2} mobilization through activation of the Gq/11-phospholipase C pathway and members of the transient receptor potential canonical (TRPC) family of channels (228, 229). This raise in $[Ca^{2+}]$i activates CaMKKβ, which in turn, activates AMPK (228, 230, 231). Hence, it is tentative to suggest that activation of PARs by coagulation factors induce AMPK activation and thus initiate autophagy (**Figure 6**).

Within ECs, the autophagy machinery is associated with vWF and P-selectin secretion in autophagic vacuoles (106, 232, 232). The primary function of vWF is to create a cell-surface adhesion site for coagulation factor VIII or platelet adhesion (among other proteins) at the endothelium membrane. Upon vascular damage, secretory granules called Weibel-Palade Bodies (WPBs), which contain thrombosis promoting factors, are assembled into chains that bind to adjacent connective tissue and in turn trap circulating platelets (234). Notably, Torisu et al. showed that autophagosomes contain vWF and that pharmacological inhibition of the autophagy or knockdown of *Atg5* or *Atg7* inhibits vWF secretion (106). Accordingly, EC-specific KO of *Atg5* or *Atg7* increased mice bleeding time (106). However, the size and composition of thrombus did not vary in EC specific *Atg5*-KO mice (235). Moreover, the secretion of vWF and P-selectin in response to the ox-LDL pro-thrombotic stimulus is associated with a decrease in Sirt1/FoxO1 signaling, and therefore autophagic flux. Moreover, the increased release of vWF and P-selectin is mediated by the inhibition of the Sirt1/

FoxO1 pathway that depresses the fusion of the autophagosome with the lysosome, thus favoring the secretion of these factors (**Figure 6**) (232).

Interestingly, it has been observed in cervical and breast cancer cell lines that decreased autophagosome-lysosome fusion is associated with increased release of extracellular nanovesicles, suggesting that extracellular secretion might provide a supplementary pathway to maintain cellular homeostasis when the autophagy degradative pathway is damaged (109). Moreover, extracellular vesicle secretion may contribute to tumor progression and metastasis (110, 111). Indeed, exosome secretion is shown to be essential for directional and efficient migration of HT1080 fibrosarcoma cells (236). Thus, these examples suggest a possible crosstalk between degradative and secretory autophagy pathways to maintain cellular homeostasis and tumor cell survival (99).

Within the TME, endothelial autophagy regulation of coagulative factors may contribute to cancer progression and cancer-related thrombosis. Cancer cells promote the secretion of WPBs and vWF from ECs (237, 238) and elevated plasmatic vWF correlate with tumor grade and metastasis (239). Moreover, vWF secretion has been reported to contribute to the process of EMT (240), the secretion of pro-inflammatory cytokines, and vascular permeability (234). Several studies show vWF-dependent cancer cell adhesion to the endothelium is mediated by integrin receptors and facilitates extravasation during metastasis (239, 241–243). It is tempting to speculate that autophagy plays a central role in mediating cancer-related thrombosis and metastasis by regulating vWF release from the activated endothelium.

Autophagy also possesses a role in the constitutive recycling of PAR1, a pivotal process to maintain the receptor pool and enable re-sensitization to its potential coagulation cascade agonists. Rab11A and Rab11B are involved in autophagosome formation by regulating membrane transport from recycling endosomes (52). Grimsey and collaborators found that under basal conditions PAR1 is constitutively internalized and recycled back to the cell surface by a Rab11B-dependent pathway, whereas Rab11A regulates PAR1 basal lysosomal degradation (244). Interestingly, when recycling is disrupted in Rab11B-deficient cells, PAR1 is sorted from endosomes to autophagosomes and subsequently degraded in autolysosomes, in a Rab11A and ATG5 dependent manner (244). These results support a role for Rab11A in PAR1 basal autophagosomal-lysosomal sorting. Consistently, in EC cell lines, Rab11-B depleted cells showed decreased expression of PAR1, as a sign of its increased degradation. Conversely, in Rab11A deficient ECs, PAR1 protein expression was elevated. Thus, Rab11B and Rab11A serve distinct functions and regulate PAR1 recycling or basal autophagic/lysosomal degradation, respectively (244).

Autophagy may regulate the signaling pathways of different coagulation factors and their appropriate cellular responses in ECs by altering the recycling and endosomal sorting of PAR1 as well as in pro-thrombotic factors release. Furthermore, the pro-inflammatory, hypoxic and nutrient-starved state of the TME may deregulate EC autophagy, promoting coagulation and in turn paving the way for metastasis. Further experimental approaches should be applied to confirm this hypothesis.

Coagulation Cascade and Autophagy Regulate Myeloid Cell Polarization Within the Tumor Microenvironment

Autophagy and inflammation work synergistically in the TME to facilitate tumor growth and metastasis (245). Within the TME, monocytes and macrophages are essential sources of extravascular FX and TF. Notably, the synthesis of FX myeloid cells determines the Tumor-Associated Macrophage (TAM) phenotype (20). Inhibition of FXa-PAR2 signaling causes reprogramming of TAMs and attenuates the recruitment of immunosuppressive neutrophils and regulatory T cells promoting anti-tumor immunity (20). Coagulation factors could facilitate invasion and metastasis by transforming monocytes and macrophages into TAM-like cells. In the same line, monocytes and macrophages treated with FXII exhibited polarized M2 phenotypes with up-regulation of CD163, IL-10, IL-8, CCL18, CCR2, and CXCR2 (246). It has been reported that FXII and FXIIa upregulate neutrophil functions, contributing to macrophage polarization and T-cell differentiation that may contribute to cancer progression [Reviewed in (247)]. Furthermore, epithelial ovarian cancer cells exposed to conditioned medium from FXII-stimulated monocytes/macrophages showed increased invasive potential (246). In the same way, TF-FVIIa complex produced CD14 and CD163 up-regulation in monocytes in addition to an increase in the expression of IL-10, IL-8, TNF-α, CXCR2, and CCR2 (248). Moreover, co-cultures of epithelial ovarian cancer cells with TF-FVIIa stimulated monocytes increased the invasive potential (248). Additionally, THP-1 human monocytic cell line stimulated with TF or FVIIa displayed an M2-like phenotype with high levels of IL-4, IL-10, TGF-β, and TNF-α. Gastric cancer cells co-cultured with TF-stimulated TAMs also showed increased migration and invasion (249). These TF-stimulated TAMs induced VEGF and MMP-9 expression, which could promote the invasive potential and angiogenesis (249). Furthermore, co-cultivation of TF-expressing cancer cell lines with human monocytes stimulated invasive capacity, an effect inhibited by a TF neutralizing antibody (250). In non-small cell lung cancer patients with lymph node metastasis, there are reported higher levels of monocyte TF mRNA, which correlate with overall survival (251).

Interestingly, Graf et al. showed that PAR2 signaling directly regulates TAM mediated immune-evasion (19). Given that macrophage-specific deletion of FX prevented *in vitro* macrophage polarization, these observations suggest that coagulation factors contribute to cancer progression by promoting the formation of TAM-like cells. Furthermore, TAM phenotypic changes were similar in both macrophage FX-deficient mice and PAR2 mutant mice and accompanied by increased T cell infiltration, suggesting that PAR2 activation by FXa impairs anti-tumor immunity (19, 20). FXa production through TF-FVIIa leading to PAR2 activation and the formation of the TF-FVIIa-FXa-Endothelial protein C receptor

(EPCR) complex is essential in the TLR4-mediated innate immune responses [reviewed in (9)]. Moreover, the TF-FVIIa-FXa-EPCR complex selectively induces expression of the TLR3/4 signaling adaptor protein Pellino-1, the transcription factor interferon regulatory factor 8 (IRF8) and a set of interferon (IFN)-regulated genes (252).

Autophagy is also associated with macrophage phenotype and activation as the AKT pathway converges both inflammatory and metabolic signals (253). The activation of mTORC1 promotes M1 polarization (254) and in accordance autophagy modulates the activity of macrophages and their responses (255–257). In general, the induction of autophagy downstream of TLR/NF-κB promotes monocyte differentiation and M2 polarization [reviewed in (258)]. Since the coagulation cascade also interacts with TLR-NF-κB signaling promoting polarization toward an M2 phenotype, it would be interesting to evaluate if there is an interaction between autophagy and the coagulation cascade in terms of innate immune signaling within the TME.

Coagulation Cascade, Autophagy, and Neutrophil Extracellular Traps in the Tumor Microenvironment and Cancer-Associated Thrombosis

Neutrophils and platelets cooperate to enhance coagulation (259). The release of intracellular components by neutrophils promotes coagulation and this has been associated with hypercoagulability and cancer-related thrombosis (260). As clinically observed in patients with sepsis and deep vein thrombosis, the activation of platelets can induce neutrophil extracellular traps (NETs), which in turn potentiate platelet aggregation (261–263). Once again demonstrating a connection to thrombosis and cancer, NETs can also formed under conditions of inflammation (264–267). As schematized in **Figure 7**, neutrophils can translocate TF to the generated NETs, where neutrophil elastase enhances TF pro-thrombotic activity through the degradation of TFPI (9). Elastase is capable of activating PAR1 and PAR2 receptors (268, 269), which may contribute to thrombus formation by promoting platelet and EC adhesion and activation.

Interestingly, the autophagy pathway is involved in mediating the delivery of thrombogenic TF to NETs, thus promoting thrombin generation and subsequent PAR-1 signaling (270). Moreover, in neutrophils, the inclusion of TF in autophagosomes is associated with its extracellular delivery. Disruption of autophagy by addition of 3-MA or BafA1 can abrogate NET release and TF trafficking, respectively (270). Notably, autophagy-mediated NET formation has been associated with CAT (271, 272), suggesting a potential interaction between autophagy, neutrophils and platelets within TME.

In line with this hypothesis, tumor-bearing mice incapable of forming NETs display decreased platelet aggregation and decreased circulating TF (271). Moreover, under in both *in vitro* and *ex vivo* experimental conditions, the pre-treatment of neutrophils with CQ inhibited NET formation (273, 274). TF, located in acidified autophagosomes, is released into NETs upon neutrophils exposure to inflammatory stimuli. Furthermore, TF from NETs can induce both thrombin generation and platelet activation mediated by PAR1 signaling (270). These data suggest that the release of TF through NETs could cause localized activation of the coagulation cascade and subsequent PAR dependent activation of platelets and ECs.

Interestingly, neutrophils from tumor-bearing orthotropic mice have increased LC3-II expression (274). Inhibition of autophagy with CQ or genetic ablation of the Receptor for Advanced Glycation End-products (RAGE), a Class III MHC protein receptor that mediates autophagy, resulted in reduced NET formation frequency within the TME (274). Based on these findings, Boone et al., proposed that NETs are upregulated in pancreatic cancer through RAGE (274). In the same line of thinking, a separate study found that, in response to platelet-derived microparticles, neutrophils can increase autophagy, mobilization of the granule content, enhanced proteolytic activity, prolonged survival, and generation of NETs (275). Neutrophil autophagy and the generation of NETs were also blunted in the presence of a competitive inhibitor of the cytokine mediator of inflammation HMGB1 (275). These findings are in line with other studies showing that autophagy in the neutrophil is essential for the NET formation, and this process is deregulated in cancer.

TARGETING THE COAGULATION CASCADE AND AUTOPHAGY FOR CANCER TREATMENT

How can the knowledge of the interplay between coagulation and autophagy be put in practice in the oncology clinic? In truth, the axis of TF-FXa/thrombin-PAR-AKT- mTOR is already exploited in numerous treatments such as the rapamycin family of drugs, anticoagulants, metformin and certain targeted therapies. VTE is a significant cause of cancer-related death (276) and its prophylactic treatment reduces mortality, with low molecular weight heparin (an anticoagulant) often employed as the first-line treatment (7). As discussed previously, anticoagulants such as low molecular weight heparin, Rivaroxaban and Dalteparin have also demonstrated direct effects on tumor growth, immune-evasion and metastasis. It has already been speculated that the specific targeting of FXa could reduce metastasis and promote anti-tumor immunity (15, 20). It was previously reported that exogenous FXa increases melanoma metastasis to the lung, spleen and lymph nodes, together with an accumulation of intra-peritoneal fluid in a syngeneic mice model (15). Furthermore, the co-administration of the anticoagulant Dalteparin reduced the FXa- increased lung metastasis, while no metastasis was observed in other organs (15). A separate study demonstrated that the targeted deletion of this coagulation factor in myeloid cells reduced tumor progression in animal models, adding justification for pharmaceutical intervention in this pathway.

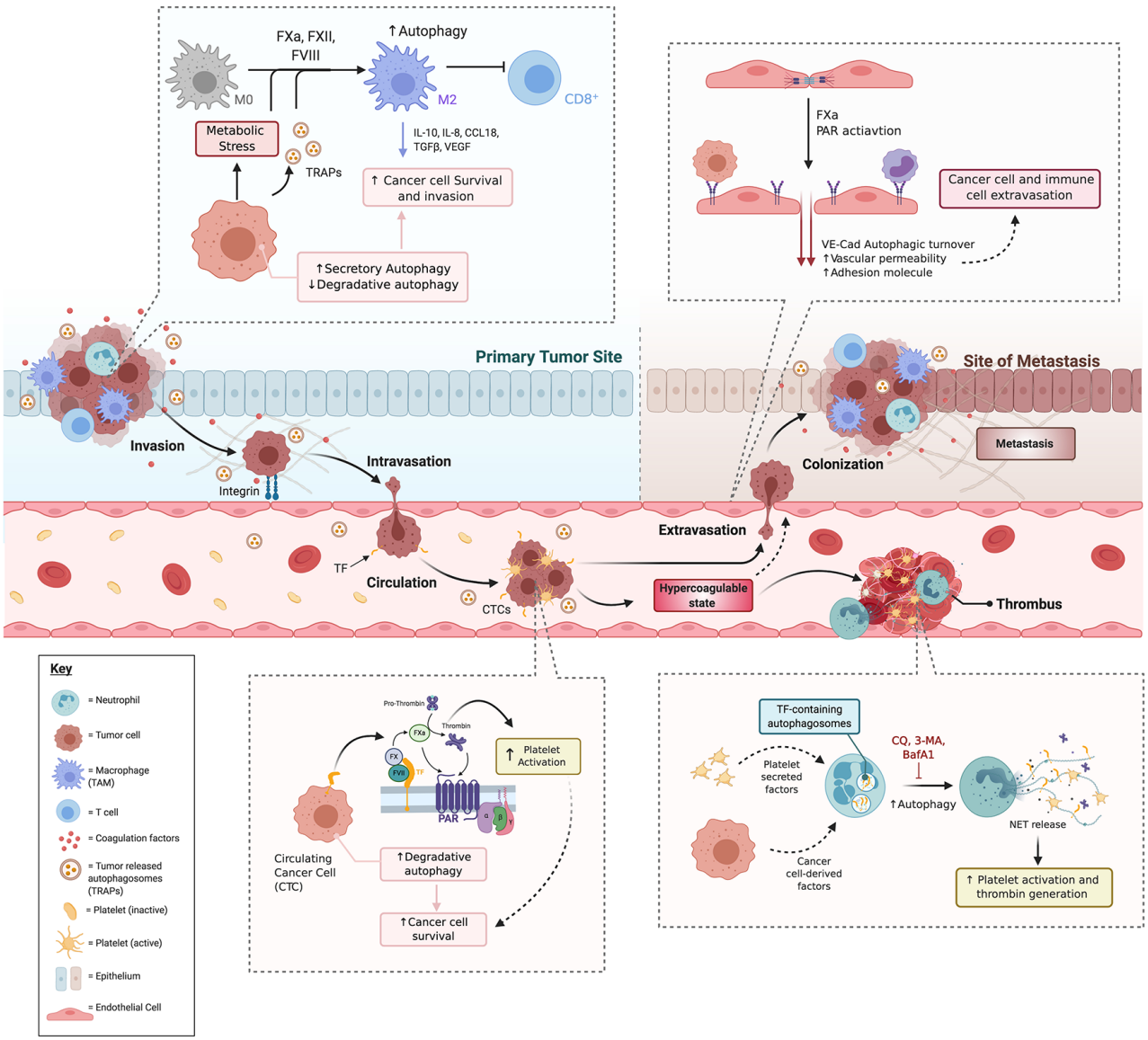


FIGURE 7 | The crosstalk between coagulation and autophagy promotes cancer-associated thrombosis and metastasis. The coagulation system is an integral part of the multifaceted approach taken by the cancer cell to survive, propagate and ultimately exhaust the body's capacity to function. We speculate that cancer cells may use common mechanisms to hijack immune cells, ECs, and platelets to integrate with the coagulation system for its own end. A common mechanism present in each of these components may be the hijacking of the autophagy pathway. At early stages, cancer cells downregulate degradative autophagy while they increase the secretion of TRAPs, indicating a disruption in the balance of degradative and secretory autophagy. This balance is influenced by the coagulation cascade, particularly, PAR activation. In turn, both TRAPs and PAR activation by coagulation factors FVII and FX promote macrophage polarization into a M2 phenotype (TAMs) with enhanced suppressive capacities, reducing cytotoxic T cell responses favoring tumor growth. PARs also promote EMT, thus enabling cancer cells to invade and intravasate. In circulation, CTCs imcrease degradative autophagy as a survival mechanism and can promote thrombogenesis through activation of the coagulation cascade on platelets, neutrophils and ECs leading to an hypercoagulable state, that also promotes metastasis through endothelial and neutrophil activation. BafA1, bafilomycin A1; CCL18, Chemokine (C-C motif) ligand 18; CQ, chloroquine; EMT, Epithelial to Mesenchymal Transition; FVII, Factor VII; FX, Factor X; FXa, activated FX; IL-8, Interleukin 8; IL-10, Interleukin 10; PAR, protease-activated receptor; TAM, tumor-associated macrophage; TF, tissue factor; TGFβ, Tumor Growth Factor beta; TRAPs, tumor cell-released autophagosomes; VE-Cad, Vascular Endothelial Cadherin; NET, Neutrophil Extracellular Trap; 3-MA, 3-methyladenine.

Furthermore, the anticoagulant Rivaroxaban has been demonstrated to give similar results to that of anti–PD-L1 therapy, and these two treatments were shown to synergize to improve anti-cancer immunity. As a mechanism, the authors propose that FXa signals through PAR2 to promote immune evasion, an effect attenuated by Rivaroxaban through the reprogramming of TAMs (20). Furthermore, co-treatment with a thrombin inhibitor, Dabigatran, and cisplatin in a model of ovarian cancer reduced tumor growth and levels of circulating activated platelets compared to Dabigatran or cisplatin alone.

Interestingly, these authors demonstrated that this co-treatment with Dabigatran promoted anti-tumor activity of cisplatin by alleviating the immunosuppressive microenvironment. Co-treatment significantly decreased the number of Myeloid-Derived Suppressor Cells (MDSCs) and dendritic cells while increasing IFN-γ production by $CD8^+$ effector T cells in ascites (277).

Similarly, in a melanoma metastasis model FXa increases endothelial permeability and promotes immune infiltration into mouse lungs, and this accumulation was reduced by the presence of the anticoagulant Daltepatin (15). These results further support the use of direct oral anticoagulants to reduce metastasis and favor an immune response against the tumor. Of note is that the inhibition of FXa on metastasis may be distinct to that of thrombin, shedding further light on the non-coagulation related roles of these coagulation factors. These observations also highlight the possibility that compounds may be developed that differentially inhibit either the coagulation or the non-coagulation actions of coagulation factors.

PARs, central proteins in the pathway connecting coagulation and in malignancy, are already a recognized cancer target (278). TF-mediated signaling *via* PAR2 has been associated with proliferation, migration and invasion of the cancer cell, and accordingly, the use of an anti-TF antibody has been shown to block PAR2 activation and suppresses tumor growth while demonstrating minimal effects on the coagulation process (279, 280). PAR inhibitors like vorapaxar, atopaxar, and PZ-128 have undergone clinical evaluation in the oncology setting. Evidence from metastatic breast cancer suggests that PAR1 blockade with PZ-128 in combination with Taxotere could be beneficial. Furthermore, a benefit may be present in dual-standard chemotherapy regimens and PZ-128 treatment in breast and ovarian cancers (278).

A further potential therapeutic target is oxidoreductase-protein disulfide isomerase (PDI) that catalyzes a thiol-disulfide exchange. In monocytes and macrophages, the activation of the TF cascade is reported to require a thiol-disulfide exchange and PDI (9). Liberated from both the EC and platelets upon agonist vascular injury, the subsequent use of PDI inhibitors could potentially attenuate platelet thrombus formation and fibrin deposition (9). Inhibition of PDI, by either antibodies or a non-specific thiol inhibitor decrease thrombus formation and fibrin generation in a mouse model of thrombosis and thus it will be interesting to examine if this could give potential benefit in the cancer setting (281).

As a central pathway for cellular function, the autophagy pathway is also a potential target for therapeutic intervention in cancer. To this end, the understanding of the action of rapamycin, discovered from a plant on Easter Island, has led to a series of "rapalogs" entering clinical oncology practice. Rapamycin, a specific mTOR inhibitor, can decrease cell migration promoted by the formation of TF-FVIIa-FXa complex in breast cancer cell model (199). Rapamycin, in combination with doxorubicin, can bring about remission in an AKT-positive lymphoma mouse model by blocking AKT signaling and overcoming chemotherapy resistance (282). Furthermore, in a vascular malformational model, the levels of D-dimer, a direct indicator of coagulation, were significantly decreased following treatment with an mTOR inhibitor (283).

Interestingly, long non-coding RNAs have been suggested to be a mechanistic target of mTOR signaling, ubiquitin-mediated proteolysis, and the coagulation cascade, opening the door to future intervention with RNA targeted therapy in cancer (284). However, as with any distribution of a ubiquitous process in cellular machinery, the use of mTOR inhibition may bring caveats. In an EC model, rapamycin strongly enhances the VEGF-induced expression of TF, possibly due to the interference in the negative feedback mechanism controlling this cycle. As VEGF is upregulated in tumors, this may explain the tumor vessel thrombosis observed in patients undergoing rapamycin therapy (285). Understanding the signaling of the coagulation system and its interaction upon cancer cell progression will be necessary for the targeted selection by the plethora of anticoagulants currently on the market to reduce tumor burden without perturbing essential hemostatic signaling.

Precision medicine based on Tyrosine Kinase Inhibitors (TKIs) has been reported to induce autophagy in many types of cancer cells (286). Noteworthy, the combination of certain TKIs with azithromycin (which dampens autophagy), enhanced cytotoxicity (286, 287). In the same line, an antibody-modified nanoparticle containing a combination of gefitinib and CQ was shown to have potential benefit in overcoming acquired EGFR-TKI resistance (288). Similarly, a combination of BafA1 with Gefitinib improved anti-tumor activity in a mouse model of triple-negative breast cancer (289). This burgeoning field may show the potential of multi-targeting to achieve both tumor-targeting selectivity and autophagy inhibition.

As previously discussed, recent studies have brought to light the immune regulatory role of coagulation components in the TME. FXa promotes immune evasion by signaling through PAR2, and the consequent addition of Rivaroxaban reprograms TAMs, supporting the translational potential of direct oral anticoagulants to overcome resistance to immunotherapy. A recent publication reported a before unidentified role of the immune checkpoint Programmed cell death protein 1 (PD-1) in regulating both lineage commitment and cell metabolism in cancer-associated myelopoiesis (290). These authors demonstrated that myeloid progenitors deficient for PD-1 manifested enhanced activation of mTORC1 in response to Granulocyte-Colony Stimulating Factor (G-CSF). This myeloid cell-specific ablation of PD-1 increased T memory cell function and anti-tumor activity. This evidence opens the door to the potential manipulation of the autophagy pathway during cancer immunotherapy.

Conclusion

The coagulation cascade and specifically its primary initiator TF possess effects that extend well beyond hemostasis and into the poorly characterized cauldron of cancer progression. Furthermore, the PARs, once believed to be exclusive mediators of the thrombin activation of platelets, have now manifested their versatility in regulating the intracellular

pathways of almost every cell type examined. At the center of every cell lies the machinery for the process of autophagy, another black hole of information, yet this cascade is known to impinge on every biological process imaginable to the minds of cell biologists and physiologists. A NCBI Pubmed search in late 2020 with the keywords coagulation, PAR, autophagy and cancer will bring back only a handful of publications. However, each word alone will offer up more references than the most diligent biomedical investigator could read in their lifetime. Therefore, it is evident that we are extensively researching each integral pathway that classically appears in our physiology textbooks. However, there is little integration between the disciplines. In this review, we made a first attempt to examine coagulation and cancer signaling pathways to try and find common ground between each cascade and in particular, identify if autophagy is at the center of the intersections. Herein, we illustrated that each pathway has components in common.

Although the functional relevance of autophagy in cancer requires further study, it is proposed that autophagy acts as a protective mechanism during cancer initiation, yet promotes later stage tumor growth and metastasis. Evidence that autophagy-associated cell death acts as an initial tumor suppressor comes from the observation that many tumors present deletions in autophagy-related genes; moreover, loss of autophagy can induce genomic instability. Conversely, autophagy could promote cancer cell survival under metabolic stress, thereby facilitating metastasis by promoting cancer cell survival. The later hypoxic, nutrient-starved and pro-inflammatory TME may deregulate local and distant autophagic pathways in ECs, platelets, immune cells, and HSCs promoting coagulation and paving the way for metastasis and thrombosis. Moreover, the knowledge that components of the autophagy machinery are required for non-conventional protein secretion of pro-inflammatory and thrombotic mediators into the TME suggests that not only conventional autophagy but also secretory pathway may have a role in CAT and metastasis. Hopefully, this first in-depth analysis of the crossovers in these differing pathways will serve to bring to light possible new areas of investigation and elucidate strategies for future therapeutic intervention.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by the grants issued by the Government of Chile: CONICYT FONDAP-15130011 (GO), Millennium Institute on Immunology & Immunotherapy IMII P09/016-F (GO), FONDECYT 1180241 (GO). The first author (CH) is a doctoral scholarship recipient of the Vice-rectory of Investigation of the Pontifical Universidad Católica de Chile. The authors (MH-C) and (CA) are recipients of CONICYT PhD fellowships (N°21151609 and 21201596, respectively). All figures were created with BioRender.com.

REFERENCES

1. Trousseau A. Phlegmasia Alba Dolens. In: . *On Clinical Medicine, Vol II*. (1873).
2. Elyamany G, Alzahrani AM, Bukhary E. Cancer-associated thrombosis: An overview. *Clin Med Insights Oncol* (2014) 8:129–37. doi: 10.4137/CMO.S18991
3. Falanga A, Russo L, Verzeroli C. Mechanisms of thrombosis in cancer. *Thromb Res* (2013) 131:S59–62. doi: 10.1016/S0049-3848(13)70024-0
4. Goldhaber SZ, Ageno W, Casella IB, Chee KH, Schellong S, Singer DE, et al. Profile of Patients Diagnosed With Acute Venous Thromboembolism in Routine Clinical Practice: The RE-COVERY DVT/PE^{TM} Study. *Am J Med* (2020) 133:936–45. doi: 10.1016/j.amjmed.2020.03.036
5. Sparsa A, Durox H, Doffoel-Hantz V, Munyangango EM, Bédane C, Cendras J, et al. High prevalence and risk factors of thromboembolism in stage IV melanoma. *J Eur Acad Dermatol Venereol* (2011) 25:340–4. doi: 10.1111/j.1468-3083.2010.03795.x
6. Kirwan CC, Descamps T, Castle J. Circulating tumour cells and hypercoagulability: a lethal relationship in metastatic breast cancer. *Clin Transl Oncol* (2020) 22:870–7. doi: 10.1007/s12094-019-02197-6
7. Ikushima S, Ono R, Fukuda K, Sakayori M, Awano N, Kondo K. Trousseau's syndrome: Cancer-associated thrombosis. *Jpn J Clin Oncol* (2016) 46:204–8. doi: 10.1093/jjco/hyv165
8. Milsom C, Rak J. Tissue factor and cancer. *Pathophysiol Haemost Thromb* (2008) 36:160–76. doi: 10.1159/000175154
9. Zelaya H, Rothmeier AS, Ruf W. Tissue factor at the crossroad of coagulation and cell signaling. *J Thromb Haemost* (2018) 16:1941–52. doi: 10.1111/jth.14246
10. Heuberger DM, Schuepbach RA. Correction to: Protease-activated receptors (PARs): Mechanisms of action and potential therapeutic modulators in PAR-driven inflammatory diseases (Thrombosis Journal (2019) 17:4. doi: 10.1186/s12959-019-0194-8). *Thromb J* (2019) 17:1–24. doi: 10.1186/s12959-019-0212-x
11. Sébert M, Sola-Tapias N, Mas E, Barreau F, Ferrand A. Protease-Activated Receptors in the Intestine: Focus on Inflammation and Cancer. *Front Endocrinol (Lausanne)* (2019) 10:717. doi: 10.3389/fendo.2019.00717
12. Driesbaugh KH, Buzza MS, Martin EW, Conway GD, Kao JPY, Antalis TM. Proteolytic activation of the protease-activated receptor (PAR)-2 by the glycosylphosphatidylinositol-anchored serine protease testisin. *J Biol Chem* (2015) 290:3529–41. doi: 10.1074/jbc.M114.628560
13. Willis Fox O, Preston RJS . Molecular basis of protease-activated receptor 1 signaling diversity. *J Thromb Haemost* (2020) 18:6–16. doi: 10.1111/jth.14643
14. Soh UJ, Dores MR, Chen B, Trejo J. Signal transduction by protease-activated receptors. *Br J Pharmacol* (2010) 160:191–203. doi: 10.1111/j.1476-5381.2010.00705.x
15. Arce M, Pinto MP, Galleguillos M, Muñoz C, Lange S, Ramirez C, et al. Coagulation factor Xa promotes solid tumor growth, experimental metastasis and endothelial cell activation. *Cancers (Basel)* (2019) 11:1103. doi: 10.3390/cancers11081103
16. Lange S, Gonzalez I, Pinto MP, Arce M, Valenzuela R, Aranda E, et al. Independent Anti-Angiogenic Capacities of Coagulation Factors X and Xa. *J Cell Physiol* (2014) 229:1673–80. doi: 10.1002/jcp.24612
17. Bae J-S, Rezaie A. Thrombin Inhibits Nuclear Factor kB and RhoA Pathways in Cytokine-stimulated Vascular Endotheliar Cells when EPCR is Occupied by Protein C. *Thromb Haemost* (2009) 101:513–20. doi: 10.1038/jid.2014.371
18. Pontarollo G, Mann A, Brandão I, Malinarich F, Schöpf M, Reinhardt C. Protease-activated receptor signaling in intestinal permeability regulation. *FEBS J* (2020) 287:645–58. doi: 10.1111/febs.15055
19. Graf C, Wilgenbus P, Kitano M, Macher-Göppinger S, Ruf W. Macrophage Factor Xa Signaling Promotes Cancer Immune Evasion. *Blood* (2018) 132:135. doi: 10.1182/blood-2018-99-119167

20. Graf C, Wilgenbus P, Pagel S, Pott J, Marini F, Reyda S, et al. Myeloid cell-synthesized coagulation factor X dampens antitumor immunity. *Sci Immunol* (2019) 4:eaaw8405. doi: 10.1126/sciimmunol.aaw8405
21. Yang Y, Stang A, Schweickert PG, Lanman NA, Paul EN, Monia BP, et al. Thrombin Signaling Promotes Pancreatic Adenocarcinoma through PAR-1-Dependent Immune Evasion. *Cancer Res* (2019) 79(13):3417–30. doi: 10.1158/0008-5472.CAN-18-3206
22. Grimsey N, Lin H, Trejo J. Endosomal signaling by protease-activated receptors. *Methods Enzymol* (2014) 535:389–401. doi: 10.1016/B978-0-12-397925-4.00022-5
23. Chen KD, Wang CC, Tsai MC, Wu CH, Yang HJ, Chen LY, et al. Interconnections between autophagy and the coagulation cascade in hepatocellular carcinoma. *Cell Death Dis* (2014) 5:e1244. doi: 10.1038/cddis.2014.212
24. Du C, Zhang T, Xiao X, Shi Y, Duan H, Ren Y. Protease-activated receptor-2 promotes kidney tubular epithelial inflammation by inhibiting autophagy via the PI3K/Akt/mTOR signalling pathway. *Biochem J* (2017) 474:2733–47. doi: 10.1042/BCJ20170272
25. Huang KT, Kuo IY, Tsai MC, Wu CH, Hsu LW, Chen LY, et al. Factor VII-Induced MicroRNA-135a Inhibits Autophagy and Is Associated with Poor Prognosis in Hepatocellular Carcinoma. *Mol Ther Nucleic Acids* (2017) 9:274–83. doi: 10.1016/j.omtn.2017.10.002
26. Baba M, Takeshige K, Baba N, Ohsumi Y. Ultrastructural analysis of the autophagic process in yeast: Detection of autophagosomes and their characterization. *J Cell Biol* (1994) 124:903–13. doi: 10.1083/jcb.124.6.903
27. Baba M, Ohsumi Y, Osumi M. Analysis of the Membrane Structures Involved in Autophagy in Yeast by Freeze-Replica Method. *Cell Struct Funct* (1995) 20:465–71. doi: 10.1247/csf.20.465
28. Nakamura N, Matsuura A, Wada Y, Ohsumi Y. Acidification of vacuoles is required for autophagic degradation in the yeast, Saccharomyces cerevisiae. *J Biochem* (1997) 121:338–44. doi: 10.1093/oxfordjournals.jbchem.a021592
29. Yang Z, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol* (2010) 22:124–31. doi: 10.1016/j.ceb.2009.11.014
30. Eskelinen EL. Autophagy: Supporting cellular and organismal homeostasis by self-eating. *Int J Biochem Cell Biol* (2019) 111:1–10. doi: 10.1016/j.biocel.2019.03.010
31. Morselli E, Galluzzi L, Kepp O, Vicencio JM, Criollo A, Maiuri MC, et al. Anti- and pro-tumor functions of autophagy. *Biochim Biophys Acta* (2009) 1793:1524–32. doi: 10.1016/j.bbamcr.2009.01.006
32. White E, DiPaola RS. The double-edged sword of autophagy modulation in cancer. *Clin Cancer Res* (2009) 15:5308–16. doi: 10.1158/1078-0432.CCR-07-5023
33. Parzych KR, Klionsky DJ. An overview of autophagy: morphology, mechanism, and regulation. *Antioxid Redox Signal* (2014) 20:460–73. doi: 10.1089/ars.2013.5371
34. Yang Y, Klionsky DJ. Autophagy and disease: unanswered questions. *Cell Death Differ* (2020) 27:858–71. doi: 10.1038/s41418-019-0480-9
35. Dower CM, Wills CA, Frisch SM, Wang HG. Mechanisms and context underlying the role of autophagy in cancer metastasis. *Autophagy* (2018) 14:1110–28. doi: 10.1080/15548627.2018.1450020
36. Galluzzi L, Pietrocola F, Bravo-San Pedro JM, Amaravadi RK, Baehrecke EH, Cecconi F, et al. Autophagy in malignant transformation and cancer progression. *EMBO J* (2015) 34:856–80. doi: 10.15252/embj.201490784
37. Hansen M, Rubinsztein DC, Walker DW. Autophagy as a promoter of longevity: insights from model organisms. *Nat Rev Mol Cell Biol* (2018) 19:579–93. doi: 10.1038/s41580-018-0033-y
38. Kaur J, Debnath J. Autophagy at the crossroads of catabolism and anabolism. *Nat Rev Mol Cell Biol* (2015) 16:461–72. doi: 10.1038/nrm4024
39. Eskelinen EL. New insights into the mechanisms of macroautophagy in mammalian cells. *Int Rev Cell Mol Biol* (2008) 266:207–47. doi: 10.1016/S1937-6448(07)66005-5
40. Kroemer G, Marino G, Levine B. Autophagy and the integrated stress response. *Mol Cell* (2010) 40:280–93. doi: 10.1016/j.molcel.2010.09.023
41. Kimmelman AC, White E. Autophagy and Tumor Metabolism. *Cell Metab* (2017) 25:1037–43. doi: 10.1016/j.cmet.2017.04.004
42. Monkkonen T, Debnath J. Inflammatory signaling cascades and autophagy in cancer. *Autophagy* (2018) 14:190–8. doi: 10.1080/15548627.2017.1345412
43. Ravanan P, Srikumar IF, Talwar P. Autophagy: The spotlight for cellular stress responses. *Life Sci* (2017) 188:53–67. doi: 10.1016/j.lfs.2017.08.029
44. Klionsky DJ, Cregg JM, Dunn WA Jr., Emr SD, Sakai Y, Sandoval IV, et al. A unified nomenclature for yeast autophagy-related genes. *Dev Cell* (2003) 5:539–45. doi: 10.1016/s1534-5807(03)00296-x
45. Tooze SA, Yoshimori T. The origin of the autophagosomal membrane. *Nat Cell Biol* (2010) 12:831–5. doi: 10.1038/ncb0910-831
46. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. *Cell* (2011) 147:728–41. doi: 10.1016/j.cell.2011.10.026
47. Reggiori F, Ungermann C. Autophagosome Maturation and Fusion. *J Mol Biol* (2017) 429:486–96. doi: 10.1016/j.jmb.2017.01.002
48. Axe EL, Walker SA, Manifava M, Chandra P, Roderick HL, Habermann A, et al. Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. *J Cell Biol* (2008) 182:685–701. doi: 10.1083/jcb.200803137
49. Polson HE, de Lartigue J, Rigden DJ, Reedijk M, Urbe S, Clague MJ, et al. Mammalian Atg18 (WIPI2) localizes to omegasome-anchored phagophores and positively regulates LC3 lipidation. *Autophagy* (2010) 6:506–22. doi: 10.4161/auto.6.4.11863
50. Reggiori F, Tucker KA, Stromhaug PE, Klionsky DJ. The Atg1-Atg13 complex regulates Atg9 and Atg23 retrieval transport from the pre-autophagosomal structure. *Dev Cell* (2004) 6:79–90. doi: 10.1016/s1534-5807(03)00402-7
51. Yamamoto H, Kakuta S, Watanabe TM, Kitamura A, Sekito T, Kondo-Kakuta C, et al. Atg9 vesicles are an important membrane source during early steps of autophagosome formation. *J Cell Biol* (2012) 198:219–33. doi: 10.1083/jcb.201202061
52. Longatti A, Lamb CA, Razi M, Yoshimura S, Barr FA, Tooze SA. TBC1D14 regulates autophagosome formation via Rab11- and ULK1-positive recycling endosomes. *J Cell Biol* (2012) 197:659–75. doi: 10.1083/jcb.201111079
53. Nakatogawa H. Mechanisms governing autophagosome biogenesis. *Nat Rev Mol Cell Biol* (2020) 21:439–58. doi: 10.1038/s41580-020-0241-0
54. Chowdhury S, Otomo C, Leitner A, Ohashi K, Aebersold R, Lander GC, et al. Insights into autophagosome biogenesis from structural and biochemical analyses of the ATG2A-WIPI4 complex. *Proc Natl Acad Sci U S A* (2018) 115:E9792–801. doi: 10.1073/pnas.1811874115
55. Maeda S, Otomo C, Otomo T. The autophagic membrane tether ATG2A transfers lipids between membranes. *Elife* (2019) 8:e45777. doi: 10.7554/eLife.45777
56. Ohsumi Y, Mizushima N. Two ubiquitin-like conjugation systems essential for autophagy. *Semin Cell Dev Biol* (2004) 15(2):231–6. doi: 10.1016/j.semcdb.2003.12.004
57. Klionsky DJ, Schulman BA. Dynamic regulation of macroautophagy by distinctive ubiquitin-like proteins. *Nat Struct Mol Biol* (2014) 21:336–45. doi: 10.1038/nsmb.2787
58. Otomo C, Metlagel Z, Takaesu G, Otomo T. Structure of the human ATG12~ATG5 conjugate required for LC3 lipidation in autophagy. *Nat Struct Mol Biol* (2013) 20:59–66. doi: 10.1038/nsmb.2431
59. Hanada T, Noda NN, Satomi Y, Ichimura Y, Fujioka Y, Takao T, et al. The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. *J Biol Chem* (2007) 282:37298–302. doi: 10.1074/jbc.C700195200
60. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* (2016) 12:1–222. doi: 10.1080/15548627.2015.1100356
61. Tanida I, Ueno T, Kominami E. Human light chain 3/MAP1LC3B is cleaved at its carboxyl-terminal Met121 to expose Gly120 for lipidation and targeting to autophagosomal membranes. *J Biol Chem* (2004) 279:47704–10. doi: 10.1074/jbc.M407016200
62. Yoshii SR, Mizushima N. Monitoring and Measuring Autophagy. *Int J Mol Sci* (2017) 18:865. doi: 10.3390/ijms18091865
63. Peng H, Yang J, Li G, You Q, Han W, Li T, et al. Ubiquitylation of p62/sequestosome1 activates its autophagy receptor function and controls selective autophagy upon ubiquitin stress. *Cell Res* (2017) 27:657–74. doi: 10.1038/cr.2017.40
64. Eskelinen EL. Maturation of autophagic vacuoles in Mammalian cells. *Autophagy* (2005) 1:1–10. doi: 10.4161/auto.1.1.1270

65. Zhao YG, Zhang H. Autophagosome maturation: An epic journey from the ER to lysosomes. *J Cell Biol* (2019) 218:757–70. doi: 10.1083/jcb.201810099
66. Nakamura S, Yoshimori T. New insights into autophagosome-lysosome fusion. *J Cell Sci* (2017) 130:1209–16. doi: 10.1242/jcs.196352
67. Jia R, Guardia CM, Pu J, Chen Y, Bonifacino JS. BORC coordinates encounter and fusion of lysosomes with autophagosomes. *Autophagy* (2017) 13:1648–63. doi: 10.1080/15548627.2017.1343768
68. Jiang P, Nishimura T, Sakamaki Y, Itakura E, Hatta T, Natsume T, et al. The HOPS complex mediates autophagosome-lysosome fusion through interaction with syntaxin 17. *Mol Biol Cell* (2014) 25:1327–37. doi: 10.1091/mbc.E13-08-0447
69. Rong Y, McPhee CK, Deng S, Huang L, Chen L, Liu M, et al. Spinster is required for autophagic lysosome reformation and mTOR reactivation following starvation. *Proc Natl Acad Sci U S A* (2011) 108:7826–31. doi: 10.1073/pnas.1013800108
70. Yu L, McPhee CK, Zheng L, Mardones GA, Rong Y, Peng J, et al. Termination of autophagy and reformation of lysosomes regulated by mTOR. *Nature* (2010) 465:942–6. doi: 10.1038/nature09076
71. Avalos Y, Canales J, Bravo-Sagua R, Criollo A, Lavandero S, Quest AF. Tumor suppression and promotion by autophagy. *BioMed Res Int* (2014) 2014:603980. doi: 10.1155/2014/603980
72. Li X, He S, Ma B. Autophagy and autophagy-related proteins in cancer. *Mol Cancer* (2020) 19:12. doi: 10.1186/s12943-020-1138-4
73. Efeyan A, Zoncu R, Sabatini DM. Amino acids and mTORC1: from lysosomes to disease. *Trends Mol Med* (2012) 18:524–33. doi: 10.1016/j.molmed.2012.05.007
74. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* (2011) 12:21–35. doi: 10.1038/nrm3025
75. Efeyan A, Zoncu R, Chang S, Gumper I, Snitkin H, Wolfson RL, et al. Regulation of mTORC1 by the Rag GTPases is necessary for neonatal autophagy and survival. *Nature* (2013) 493:679–83. doi: 10.1038/nature11745
76. Jung CH, Ro SH, Cao J, Otto NM, Kim DH. mTOR regulation of autophagy. *FEBS Lett* (2010) 584:1287–95. doi: 10.1016/j.febslet.2010.01.017
77. Alers S, Loffler AS, Wesselborg S, Stork B. Role of AMPK-mTOR-Ulk1/2 in the regulation of autophagy: cross talk, shortcuts, and feedbacks. *Mol Cell Biol* (2012) 32:2–11. doi: 10.1128/MCB.06159-11
78. Chan EY. mTORC1 phosphorylates the ULK1-mAtg13-FIP200 autophagy regulatory complex. *Sci Signal* (2009) 2:pe51. doi: 10.1126/scisignal.284pe51
79. Hosokawa N, Hara T, Kaizuka T, Kishi C, Takamura A, Miura Y, et al. Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. *Mol Biol Cell* (2009) 20:1981–91. doi: 10.1091/mbc.E08-12-1248
80. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol* (2011) 13:132–41. doi: 10.1038/ncb2152
81. Ha J, Guan KL, Kim J. AMPK and autophagy in glucose/glycogen metabolism. *Mol Aspects Med* (2015) 46:46–62. doi: 10.1016/j.mam.2015.08.002
82. Hardie DG. AMPK–sensing energy while talking to other signaling pathways. *Cell Metab* (2014) 20:939–52. doi: 10.1016/j.cmet.2014.09.013
83. Dunlop EA, Tee AR. The kinase triad, AMPK, mTORC1 and ULK1, maintains energy and nutrient homoeostasis. *Biochem Soc Trans* (2013) 41:939–43. doi: 10.1042/BST20130030
84. Chavez-Dominguez R, Perez-Medina M, Lopez-Gonzalez JS, Galicia-Velasco M, Aguilar-Cazares D. The Double-Edge Sword of Autophagy in Cancer: From Tumor Suppression to Pro-tumor Activity. *Front Oncol* (2020) 10:578418. doi: 10.3389/fonc.2020.578418
85. Choi AM, Ryter SW, Levine B. Autophagy in human health and disease. *N Engl J Med* (2013) 368:1845–6. doi: 10.1056/NEJMc1303158
86. Verzella D, Pescatore A, Capece D, Vecchiotti D, Ursini MV, Franzoso G, et al. Life, death, and autophagy in cancer: NF-κB turns up everywhere. *Cell Death Dis* (2020) 11:210. doi: 10.1038/s41419-020-2399-y
87. Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, et al. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* (2005) 122:927–39. doi: 10.1016/j.cell.2005.07.002
88. Singh SS, Vats S, Chia AY, Tan TZ, Deng S, Ong MS, et al. Dual role of autophagy in hallmarks of cancer. *Oncogene* (2018) 37:1142–58. doi: 10.1038/s41388-017-0046-6
89. Son Y, Cheong YK, Kim NH, Chung HT, Kang DG, Pae HO. Mitogen-Activated Protein Kinases and Reactive Oxygen Species: How Can ROS Activate MAPK Pathways? *J Signal Transduct* (2011) 2011:792639. doi: 10.1155/2011/792639
90. Galluzzi L, Bravo-San Pedro JM, Levine B, Green DR, Kroemer G. Pharmacological modulation of autophagy: therapeutic potential and persisting obstacles. *Nat Rev Drug Discov* (2017) 16:487–511. doi: 10.1038/nrd.2017.22
91. Pan T, Rawal P, Wu Y, Xie W, Jankovic J, Le W. Rapamycin protects against rotenone-induced apoptosis through autophagy induction. *Neuroscience* (2009) 164:541–51. doi: 10.1016/j.neuroscience.2009.08.014
92. Petiot A, Ogier-Denis E, Blommaart EF, Meijer AJ, Codogno P. Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. *J Biol Chem* (2000) 275:992–8. doi: 10.1074/jbc.275.2.992
93. Galluzzi L, Baehrecke EH, Ballabio A, Boya P, Bravo-San Pedro JM, Cecconi F, et al. Molecular definitions of autophagy and related processes. *EMBO J* (2017) 36:1811–36. doi: 10.15252/embj.201796697
94. Mauvezin C, Neufeld TP. Bafilomycin A1 disrupts autophagic flux by inhibiting both V-ATPase-dependent acidification and Ca-P60A/SERCA-dependent autophagosome-lysosome fusion. *Autophagy* (2015) 11:1437–8. doi: 10.1080/15548627.2015.1066957
95. Pasquier B. Autophagy inhibitors. *Cell Mol Life Sci* (2016) 73:985–1001. doi: 10.1007/s00018-015-2104-y
96. Cadwell K, Debnath J. Beyond self-eating: The control of nonautophagic functions and signaling pathways by autophagy-related proteins. *J Cell Biol* (2018) 217:813–22. doi: 10.1083/jcb.201706157
97. Deretic V, Levine B. Autophagy balances inflammation in innate immunity. *Autophagy* (2018) 14:243–51. doi: 10.1080/15548627.2017.1402992
98. New J, Thomas SM. Autophagy-dependent secretion: mechanism, factors secreted, and disease implications. *Autophagy* (2019) 15:1682–93. doi: 10.1080/15548627.2019.1596479
99. Gonzalez CD, Resnik R, Vaccaro MI. Secretory Autophagy and Its Relevance in Metabolic and Degenerative Disease. *Front Endocrinol* (2020) 11:266. doi: 10.3389/fendo.2020.00266
100. Jiang S, Dupont N, Castillo EF, Deretic V. Secretory versus degradative autophagy: unconventional secretion of inflammatory mediators. *J Innate Immun* (2013) 5:471–9. doi: 10.1159/000346707
101. Galluzzi L, Green DR. Autophagy-Independent Functions of the Autophagy Machinery. *Cell* (2019) 177:1682–99. doi: 10.1016/j.cell.2019.05.026
102. New J, Arnold L, Ananth M, Alvi S, Thornton M, Werner L, et al. Secretory Autophagy in Cancer-Associated Fibroblasts Promotes Head and Neck Cancer Progression and Offers a Novel Therapeutic Target. *Cancer Res* (2017) 77:6679–91. doi: 10.1158/0008-5472.CAN-17-1077
103. Pettersen K, Andersen S, Degen S, Tadini V, Grosjean J, Hatakeyama S, et al. Cancer cachexia associates with a systemic autophagy-inducing activity mimicked by cancer cell-derived IL-6 trans-signaling. *Sci Rep* (2017) 7:2046. doi: 10.1038/s41598-017-02088-2
104. Dupont N, Jiang S, Pilli M, Ornatowski W, Bhattacharya D, Deretic V. Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1beta. *EMBO J* (2011) 30:4701–11. doi: 10.1038/emboj.2011.398
105. Thorburn J, Horita H, Redzic J, Hansen K, Frankel AE, Thorburn A. Autophagy regulates selective HMGB1 release in tumor cells that are destined to die. *Cell Death Differ* (2009) 16:175–83. doi: 10.1038/cdd.2008.143
106. Torisu T, Torisu K, Lee IH, Liu J, Malide D, Combs CA, et al. Autophagy regulates endothelial cell processing, maturation and secretion of von Willebrand factor. *Nat Med* (2013) 19:1281–7. doi: 10.1038/nm.3288
107. Zahoor M, Farhan H. Crosstalk of Autophagy and the Secretory Pathway and Its Role in Diseases. *Int Rev Cell Mol Biol* (2018) 337:153–84. doi: 10.1016/bs.ircmb.2017.12.004
108. Lock R, Kenific CM, Leidal AM, Salas E, Debnath J. Autophagy-dependent production of secreted factors facilitates oncogenic RAS-driven invasion. *Cancer Discov* (2014) 4:466–79. doi: 10.1158/2159-8290.CD-13-0841
109. Wang K, Wei Y, Liu W, Liu L, Guo Z, Fan C, et al. Mechanical Stress-Dependent Autophagy Component Release via Extracellular Nanovesicles in Tumor Cells. *ACS Nano* (2019) 13:4589–602. doi: 10.1021/acsnano.9b00587

110. Zhang L, Zhang S, Yao J, Lowery FJ, Zhang Q, Huang WC, et al. Microenvironment-induced PTEN loss by exosomal microRNA primes brain metastasis outgrowth. *Nature* (2015) 527:100–4. doi: 10.1038/nature15376
111. Zomer A, Maynard C, Verweij FJ, Kamermans A, Schafer R, Beerling E, et al. In Vivo imaging reveals extracellular vesicle-mediated phenocopying of metastatic behavior. *Cell* (2015) 161:1046–57. doi: 10.1016/j.cell.2015.04.042
112. Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, et al. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell* (2006) 10:51–64. doi: 10.1016/j.ccr.2006.06.001
113. Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G, Chen HY, et al. Autophagy Suppresses Tumorigenesis through Elimination of p62. *Cell* (2009) 137:1062–75. doi: 10.1016/j.cell.2009.03.048
114. Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. *Nat Rev Cancer* (2007) 7:961–7. doi: 10.1038/nrc2254
115. Lorin S, Hamaï A, Mehrpour M, Codogno P. Autophagy regulation and its role in cancer. *Semin Cancer Biol* (2013) 23:361–79. doi: 10.1016/j.semcancer.2013.06.007
116. Remiker AS, Palumbo JS. Mechanisms coupling thrombin to metastasis and tumorigenesis. *Thromb Res* (2018) 164:S29–33. doi: 10.1016/j.thromres.2017.12.020
117. Henrikson KP, Salazar SL, Fenton JW, Pentecost BT. Role of thrombin receptor in breast cancer invasiveness. *Br J Cancer* (1999) 79:401–6. doi: 10.1038/sj.bjc.6690063
118. Dawood S, Austin L, Cristofanilli M. Cancer stem cells: implications for cancer therapy. *Oncol (Williston Park)* (2014) 28:1101–7, 1110.
119. Orellana R, Kato S, Erices R, Bravo ML, Gonzalez P, Oliva B, et al. Platelets enhance tissue factor protein and metastasis initiating cell markers, and act as chemoattractants increasing the migration of ovarian cancer cells. *BMC Cancer* (2015) 15:290. doi: 10.1186/s12885-015-1304-z
120. Shaker H, Harrison H, Clarke R, Landberg G, Bundred NJ, Versteeg HH, et al. Tissue Factor promotes breast cancer stem cell activity in vitro. *Oncotarget* (2017) 8:25915–27. doi: 10.18632/oncotarget.13928
121. Hattori T, Kawano S, Tanaka S, Matsubara R, Sakamoto T, Hashiguchi Y, et al. Elevated expression of protease-activated receptor 1 via DNp63 downregulation contributes to nodal metastasis in oral squamous cell carcinoma. *Int J Oral Maxillofac Surg* (2020) S0901–5027(20):30197–1. doi: 10.1016/j.ijom.2020.04.021
122. Rydén L, Grabau D, Schaffner F, Jönsson PE, Ruf W, Belting M. Evidence for tissue factor phosphorylation and its correlation with protease-activated receptor expression and the prognosis of primary breast cancer. *Int J Cancer* (2010) 126:2330–40. doi: 10.1002/ijc.24921
123. Grozovsky R, Giannini S, Falet H, Hoffmeister KM. Regulating billions of blood platelets: Glycans and beyond. *Blood* (2015) 126:1877–84. doi: 10.1182/blood-2015-01-569129
124. Behrens K, Alexander WS. Cytokine control of megakaryopoiesis. *Growth Factors* (2018) 36:89–103. doi: 10.1080/08977194.2018.1498487
125. Geddis AE. Megakayopoiesis. *Semin Hematol* (2010) 47:212–9. doi: 10.1053/j.seminhematol.2010.03.001.Megakaryopoiesis
126. Geddis AE, Fox NE, Kaushansky K. Phosphatidylinositol 3-Kinase Is Necessary but Not Sufficient for Thrombopoietin-induced Proliferation in Engineered Mpl-bearing Cell Lines as Well as in Primary Megakaryocytic Progenitors. *J Biol Chem* (2001) 276:34473–9. doi: 10.1074/jbc.M105178200
127. Guo T, Wang X, Qu Y, Yin Y, Jing T, Zhang Q. Megakaryopoiesis and platelet production: insight into hematopoietic stem cell proliferation and differentiation. *Stem Cell Invest* (2015) 2:3. doi: 10.3978/j.issn.2306-9759.2015.02.01
128. Matsumura I, Nakajima K, Wakao H, Hattori S, Hashimoto K, Sugahara H, et al. Involvement of Prolonged Ras Activation in Thrombopoietin-Induced Megakaryocytic Differentiation of a Human Factor-Dependent Hematopoietic Cell Line. *Mol Cell Biol* (1998) 18:4282–90. doi: 10.1128/mcb.18.7.4282
129. Colosetti P, Puissant A, Robert G, Luciano F, Jacquel A, Gounon P, et al. Autophagy is an important event for megakaryocytic differentiation of the chronic myelogenous leukemia K562 cell line. *Autophagy* (2009) 5:1092–8. doi: 10.4161/auto.5.8.9889
130. Raslova H, Baccini V, Loussaief L, Comba B, Larghero J, Debili N, et al. Mammalian target of rapamycin (mTOR) regulates both proliferation of megakaryocyte progenitors and late stages of megakaryocyte differentiation. *Blood* (2006) 107:2303–10. doi: 10.1182/blood-2005-07-3005
131. Saliba J, Saint-Martin C, Di Stefano A, Lenglet G, Marty C, Keren B, et al. Germline duplication of ATG2B and GSKIP predisposes to familial myeloid malignancies. *Nat Genet* (2015) 47:1131–40. doi: 10.1038/ng.3380
132. Kozuma Y, Yuki S, Ninomiya H, Nagasawa T, Kojima H. Caspase activation is involved in early megakaryocyte differentiation but not in platelet production from megakaryocytes. *Leukemia* (2009) 23:1080–6. doi: 10.1038/leu.2009.7
133. Cleyrat C, Darehshouri A, Steinkamp M, Vilaine M, Boassa B, Ellisman M, et al. Mpl traffics to the cell surface through conventional and unconventional routes. *Traffic* (2014) 15:961–82. doi: 10.1161/CIRCULATIONAHA.110.956839
134. Hitchcock IS, Chen MM, King JR, Kaushansky K. YRRL motifs in the cytoplasmic domain of the thrombopoietin receptor regulate receptor internalization and degradation. *Blood* (2008) 112:2222–31. doi: 10.1182/blood-2008-01-134049
135. Valet C, Levade M, Chicanne G, Bilanges B, Cabou C, Viaud J, et al. A dual role for the class III PI3K, Vps34, in platelet production and thrombus growth. *Blood* (2017) 130:2032–42. doi: 10.1182/blood-2017-04-781641
136. Bertović I, Kurelić R, Milošević I, Bender M, Krauss M, Haucke V, et al. Vps34 derived phosphatidylinositol 3-monophosphate modulates megakaryocyte maturation and proplatelet production through late endosomes/lysosomes. *J Thromb Haemost* (2020), 18:1–17. doi: 10.1111/jth.14764
137. Guerriero R, Parolini I, Testa U, Samoggia P, Petrucci E, Sargiacomo M, et al. Inhibition of TPO-induced MEK or mTOR activity induces opposite effects on the ploidy of human differentiating megakaryocytes. *J Cell Sci* (2006) 119:744–52. doi: 10.1242/jcs.02784
138. Cao Y, Cai J, Zhang S, Yuan N, Li X, Fang Y, et al. Loss of autophagy leads to failure in megakaryopoiesis, megakaryocyte differentiation, and thrombopoiesis in mice. *Exp Hematol* (2015) 43:488–94. doi: 10.1016/j.exphem.2015.01.001
139. Ouseph MM, Huang Y, Banerjee M, Joshi S, MacDonald L, Zhong Y, et al. Autophagy is induced upon platelet activation and is essential for hemostasis and thrombosis. *Blood* (2015) 126:1224–33. doi: 10.1182/blood-2014-09-598722
140. Cao Y, Zhang S, Yuan N, Wang J, Li X, Xu F, et al. Hierarchal autophagic divergence of hematopoietic system. *J Biol Chem* (2015) 290:23050–63. doi: 10.1074/jbc.M115.650028
141. Thompson CB, Jakubowski JA. The pathophysiology and clinical relevance of platelet heterogeneity. *Blood* (1988) 72:1–8. doi: 10.1182/blood.v72.1.1.bloodjournal7211
142. Kaser A, Brandacher G, Steurer W, Kaser S, Offner FA, Zoller H, et al. Interleukin-6 stimulates thrombopoiesis through thrombopoietin: Role in inflammatory thrombocytosis. *Blood* (2001) 98:2720–5. doi: 10.1182/blood.V98.9.2720
143. Kirito K, Osawa M, Morita H, Shimizu R, Yamamoto M, Oda A, et al. A functional role of Stat3 in in vivo megakaryopoiesis. *Blood* (2002) 99:3220–7. doi: 10.1182/blood.V99.9.3220
144. Haas S, Hansson J, Klimmeck D, Loeffler D, Velten L, Uckelmann H, et al. Inflammation-Induced Emergency Megakaryopoiesis Driven by Hematopoietic Stem Cell-like Megakaryocyte Progenitors. *Cell Stem Cell* (2015) 17:422–34. doi: 10.1016/j.stem.2015.07.007
145. Nishimura S, Nagasaki M, Kunishima S, Sawaguchi A, Sakata A, Sakaguchi H, et al. IL-1α induces thrombopoiesis through megakaryocyte rupture in response to acute platelet needs. *J Cell Biol* (2015) 209:453–66. doi: 10.1083/jcb.201410052
146. Lewis JC, Maldonado JE, Mann KG, Moertel CG. Ultrastructural cytochemistry of platelets and megakaryocytes in the carcinoid syndrome. *Mayo Clin Proc* (1976) 51:585—593.
147. Banerjee M, Huang Y, Ouseph MM, Joshi S, Pokrovskaya I, Storrie B, et al. Autophagy in Platelets. *Methods Mol Biol* (2019) 1880:511–28. doi: 10.1007/978-1-4939-8873-0_32
148. Kuma A, Mizushima N. Chromosomal mapping of the GFP-LC3 transgene in GFP-LC3 mice. *Autophagy* (2008) 4:61–2. doi: 10.4161/auto.4846
149. Arsov I, Li X, Matthews G, Coradin J, Hartmann B, Simon AK, et al. BAC-mediated transgenic expression of fluorescent autophagic protein Beclin 1 reveals a role for Beclin 1 in lymphocyte development. *Cell Death Differ* (2008) 15:1385–95. doi: 10.1038/cdd.2008.59

150. Rowley JW, Oler AJ, Tolley NDO. Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes (Blood (2011) 118, 14 (e101-e111)). *Blood* (2014) 123:3843. doi: 10.1182/blood-2011-03-339705.An
151. Feng W, Chang C, Luo D, Su H, Yu S, Hua W, et al. Dissection of autophagy in human platelets. *Autophagy* (2014) 10:642–51. doi: 10.4161/auto.27832
152. Lee SH, Du J, Stitham J, Atteya G, Lee S, Xiang Y, et al. Inducing mitophagy in diabetic platelets protects against severe oxidative stress. *EMBO Mol Med* (2016) 8:779–95. doi: 10.15252/emmm.201506046
153. Liu Y, Hu M, Luo D, Yue M, Wang S, Chen X, et al. Class III PI3K positively regulates platelet activation and thrombosis via PI(3)P-directed function of NADPH oxidase. *Arterioscler Thromb Vasc Biol* (2017) 37:2075–86. doi: 10.1161/ATVBAHA.117.309751
154. Menter DG, Kopetz S, Hawk E, Sood AK, Loree JM, Gresele P, et al. Platelet "first responders" in wound response, cancer, and metastasis. *Cancer Metastasis Rev* (2017) 36:199–213. doi: 10.1007/s10555-017-9682-0
155. Nieswandt B, Varga-Szabo D, Elvers M. Integrins in platelet activation. *J Thromb Haemost* (2009) 206–9. doi: 10.1111/j.1538-7836.2009.03370.x
156. Wang X, Fu YF, Liu X, Feng G, Xiong D, Mu GF, et al. ROS Promote ox-LDL-induced platelet activation by up-regulating autophagy through the inhibition of the PI3K/AKT/mTOR pathway. *Cell Physiol Biochem* (2018) 50:1779–93. doi: 10.1159/000494795
157. Zhang W, Ren H, Xu C, Zhu C, Wu H, Liu D, et al. Hypoxic mitophagy regulates mitochondrial quality and platelet activation and determines severity of I/R heart injury. *Elife* (2016) 5:1–20. doi: 10.7554/eLife.21407
158. Kania E, Pajak B, Orzechowski A. Calcium homeostasis and ER stress in control of autophagy in cancer cells. *BioMed Res Int* (2015) 2015:352794. doi: 10.1155/2015/352794
159. Grotemeier A, Alers S, Pfisterer SG, Paasch F, Daubrawa M, Dieterle A, et al. AMPK-independent induction of autophagy by cytosolic Ca2+ increase. *Cell Signal* (2010) 22:914–25. doi: 10.1016/j.cellsig.2010.01.015
160. Zhu X, Chen Y, Cui Y. Absolute Neutrophil Count and Mean Platelet Volume in the Blood as Biomarkers to Detect Lung Cancer. *Dis Markers* (2020) 2020):1371964. doi: 10.1155/2020/1371964
161. Rachidi S, Kaur M, Lautenschlaeger T, Li Z. Platelet count correlates with stage and predicts survival in melanoma. *Platelets* (2019) 30:1042–6. doi: 10.1080/09537104.2019.1572879
162. St-Onge M, Lagarde S, Laflamme C, Rollet-Labelle E, Marois L, Naccache PH, et al. Proteinase-activated receptor-2 up-regulation by Fcγ-receptor activation in human neutrophils. *FASEB J* (2010) 24:2116–25. doi: 10.1096/fj.09-146167
163. Labelle M, Begum S, Hynes RO. Direct Signaling between Platelets and Cancer Cells Induces an Epithelial-Mesenchymal-Like Transition and Promotes Metastasis. *Cancer Cell* (2011) 20:576–90. doi: 10.1016/j.ccr.2011.09.009
164. Labelle M, Begum S, Hynes RO. Platelets guide the formation of early metastatic niches. *Proc Natl Acad Sci U S A* (2014) 111:E3053–61. doi: 10.1073/pnas.1411082111
165. Erices R, Cubillos S, Aravena R, Santoro F, Marquez M, Orellana R, et al. Diabetic concentrations of metformin inhibit platelet-mediated ovarian cancer cell progression. *Oncotarget* (2017) 8:20865–80. doi: 10.18632/oncotarget.15348
166. Josa V, Ferenczi S, Szalai R, Fuder E, Kuti D, Horvath K, et al. Thrombocytosis and E ff ects of IL-6 Knock-Out in a Colitis-Associated Cancer Model. *Int J Mol Sci* (2020) 21(17):6218. doi: 10.3390/ijms21176218
167. Avecilla ST, Hattori K, Heissig B, Tejada R, Liao F, Shido K, et al. Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nat Med* (2004) 10:64–71. doi: 10.1038/nm973
168. Casella I, Feccia T, Chelucci C, Samoggia P, Castelli G, Guerriero R, et al. Autocrine-paracrine VEGF loops potentiate the maturation of megakaryocytic precursors through Flt1 receptor. *Blood* (2003) 101:1316–23. doi: 10.1182/blood-2002-07-2184
169. Pitchford SC, Lodie T, Rankin SM. VEGFR1 stimulates a CXCR4-dependent translocation of megakaryocytes to the vascular niche, enhancing platelet production in mice. *Blood* (2012) 120:2787–95. doi: 10.1182/blood-2011-09-378174
170. Coppola S, Narciso L, Feccia T, Bonci D, Calabrò L, Morsilli O, et al. Enforced expression of KDR receptor promotes proliferation, survival and megakaryocytic differentiation of TF1 progenitor cell line. *Cell Death Differ* (2006) 13:61–74. doi: 10.1038/sj.cdd.4401698
171. Monlish DA, Bhatt ST, Schuettpelz LG. The role of toll-like receptors in hematopoietic malignancies. *Front Immunol* (2016) 7:390. doi: 10.3389/fimmu.2016.00390
172. Ge Y, Huang M, Yao YM. ming. Autophagy and proinflammatory cytokines: Interactions and clinical implications. *Cytokine Growth Factor Rev* (2018) 43:38–46. doi: 10.1016/j.cytogfr.2018.07.001
173. Lippitz BE. Cytokine patterns in patients with cancer: A systematic review. *Lancet Oncol* (2013) 14:e218–28. doi: 10.1016/S1470-2045(12)70582-X
174. Manfredi AA, Baldini M, Camera M, Baldissera E, Brambilla M, Peretti G, et al. Anti-TNFα agents curb platelet activation in patients with rheumatoid arthritis. *Ann Rheum Dis* (2016) 75:1511–20. doi: 10.1136/annrheumdis-2015-208442
175. Kojok K, El Akoum S, Mohsen M, Mourad W, Merhi Y. CD40L priming of platelets via NF-κB activation is CD40-and TAK1-dependent. *J Am Heart Assoc* (2018) 7:eo3677. doi: 10.1161/JAHA.118.009636
176. Lumadue JA, Lanzkron SM, Kennedy SD, Kuhl DT. K.T.S. Cytokine Induction of Platelet Activation. *Am J Clin Pathol* (1996) 106:795–8. doi: 10.1017/CBO9781107415324.004
177. You L, Wang Z, Li H, Shou J, Jing Z, Xie J, et al. The role of STAT3 in autophagy. *Autophagy* (2015) 11:729–39. doi: 10.1080/15548627.2015.1017192
178. Caiqi MA, Jinye XIE, Chuanghua LUO, Haofan YIN, Ruopu LI, Wang X, et al. OxLDL promotes lymphangiogenesis and lymphatic metastasis in gastric cancer by upregulating VEGF-C expression and secretion. *Int J Oncol* (2019) 54:572–84. doi: 10.3892/ijo.2018.4648
179. Wan F, Qin X, Zhang G, Lu X, Zhu Y, Zhang H, et al. Oxidized low-density lipoprotein is associated with advanced-stage prostate cancer. *Tumour Biol* (2015) 36:3573–82. doi: 10.1007/s13277-014-2994-6
180. Delimaris I, Faviou E, Antonakos G, Stathopoulou E, Zachari A, Dionyssiou-Asteriou A, et al. serum oxidizability and serum lipid levels in patients with breast or ovarian cancer. *Clin Biochem* (2007) 40:1129–34. doi: 10.1016/j.clinbiochem.2007.06.007
181. Timp JF, Braekkan SK, Versteeg HH, Cannegieter SC. Epidemiology of cancer-associated venous thrombosis. *Blood* (2013) 122:1712–23. doi: 10.1182/blood-2013-04-460121
182. Chen M, Kakutani M, Masaki T, Sawamura T, Chen M, Narumiya S, et al. Activation-dependent surface expression of LOX-1 in human platelets. *Biochem Biophys Res Commun* (2001) 282:153–8. doi: 10.1006/bbrc.2001.4516
183. Geddings JE, Hisada Y, Boulaftali Y, Getz TM, Whelihan M, Fuentes R, et al. Tissue factor-positive tumor microvesicles activate platelets and enhance thrombosis in mice. *J Thromb Haemost* (2016) 14:153–66. doi: 10.1111/jth.13181
184. Chen KD, Lin CC, Tsai MC, Huang KT, Chiu KW. Tumor microenvironment mediated by suppression of autophagic flux drives liver malignancy. *BioMed J* (2018) 41:163–8. doi: 10.1016/j.bj.2018.03.002
185. Liu J, Xia H, Kim M, Xu L, Li Y, Zhang L, et al. Beclin1 controls the levels of p53 by regulating the deubiquitination activity of USP10 and USP13. *Cell* (2011) 147:223–34. doi: 10.1016/j.cell.2011.08.037
186. Beauchamp EM, Platanias LC. The evolution of the TOR pathway and its role in cancer. *Oncogene* (2013) 32:3923–32. doi: 10.1038/onc.2012.567
187. Dunlop EA, Tee AR. mTOR and autophagy: a dynamic relationship governed by nutrients and energy. *Semin Cell Dev Biol* (2014) 36:121–9. doi: 10.1016/j.semcdb.2014.08.006
188. Dang CV. Links between metabolism and cancer. *Genes Dev* (2012) 26:877–90. doi: 10.1101/gad.189365.112
189. Howell JJ, Manning BD. mTOR couples cellular nutrient sensing to organismal metabolic homeostasis. *Trends Endocrinol Metab* (2011) 22:94–102. doi: 10.1016/j.tem.2010.12.003
190. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Sci (80-)* (2009) 324:1029–33. doi: 10.1126/science.1160809
191. Love NK, Keshavan N, Lewis R, Harris WA, Agathocleous MA. nutrient-sensitive restriction point is active during retinal progenitor cell differentiation. *Development* (2014) 141:697–706. doi: 10.1242/dev.103978
192. Chang Q, Jurisica I, Do T, Hedley DW. Hypoxia predicts aggressive growth and spontaneous metastasis formation from orthotopically grown primary xenografts of human pancreatic cancer. *Cancer Res* (2011) 71:3110–20. doi: 10.1158/0008-5472.CAN-10-4049

193. Chang LH, Chen CH, Huang DY, Pai HC, Pan SL, Teng CM. Thrombin induces expression of twist and cell motility via the hypoxia-inducible factor-1alpha translational pathway in colorectal cancer cells. *J Cell Physiol* (2011) 226:1060–8. doi: 10.1002/jcp.22428
194. Rankin EB, Giaccia AJ. The role of hypoxia-inducible factors in tumorigenesis. *Cell Death Differ* (2008) 15:678–85. doi: 10.1038/cdd.2008.21
195. Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* (2003) 3:721–32. doi: 10.1038/nrc1187
196. Sun Z, Cao B, Wu J. Protease-activated receptor 2 enhances renal cell carcinoma cell invasion and migration via PI3K/AKT signaling pathway. *Exp Mol Pathol* (2015) 98:382–9. doi: 10.1016/j.yexmp.2015.03.018
197. Tang KL, Tang HY, Du Y, Tian T, Xiong SJ. PAR-2 promotes cell proliferation, migration, and invasion through activating PI3K/AKT signaling pathway in oral squamous cell carcinoma. *Biosci Rep* (2019) 39:721–32. doi: 10.1042/BSR20182476
198. Jiang X, Guo YL, Bromberg ME. Formation of tissue factor-factor VIIa-factor Xa complex prevents apoptosis in human breast cancer cells. *Thromb Haemost* (2006) 96:196–201. doi: 10.1160/TH06-01-0057
199. Jiang X, Zhu S, Panetti TS, Bromberg ME. Formation of tissue factor-factor VIIa-factor Xa complex induces activation of the mTOR pathway which regulates migration of human breast cancer cells. *Thromb Haemost* (2008) 100:127–33. doi: 10.1160/TH07-12-0722
200. Zhang X, Shi H, Yuan X, Jiang P, Qian H, Xu W. Tumor-derived exosomes induce N2 polarization of neutrophils to promote gastric cancer cell migration. *Mol Cancer* (2018) 17:1–16. doi: 10.1186/s12943-018-0898-6
201. Wen ZF, Liu H, Gao R, Zhou M, Ma J, Zhang Y, et al. Tumor cell-released autophagosomes (TRAPs) promote immunosuppression through induction of M2-like macrophages with increased expression of PD-L1. *J Immunother Cancer* (2018) 6:1–16. doi: 10.1186/s40425-018-0452-5
202. Aasen T, Leithe E, Graham SV, Kameritsch P, Mayan MD, Mesnil M, et al. Connexins in cancer: bridging the gap to the clinic. *Oncogene* (2019) 38:4429–51. doi: 10.1038/s41388-019-0741-6
203. Czyz J. The stage-specific function of gap junctions during tumourigenesis. *Cell Mol Biol Lett* (2008) 13:92–102. doi: 10.2478/s11658-007-0039-5
204. Gershon E, Plaks V, Dekel N. Gap junctions in the ovary: expression, localization and function. *Mol Cell Endocrinol* (2008) 282:18–25. doi: 10.1016/j.mce.2007.11.001
205. Mitchell JA, Lye SJ. Differential activation of the connexin 43 promoter by dimers of activator protein-1 transcription factors in myometrial cells. *Endocrinology* (2005) 146:2048–54. doi: 10.1210/en.2004-1066
206. Bates DC, Sin WC, Aftab Q, Naus CC. Connexin43 enhances glioma invasion by a mechanism involving the carboxy terminus. *Glia* (2007) 55:1554–64. doi: 10.1002/glia.20569
207. Elzarrad MK, Haroon A, Willecke K, Dobrowolski R, Gillespie MN, Al-Mehdi AB. Connexin-43 upregulation in micrometastases and tumor vasculature and its role in tumor cell attachment to pulmonary endothelium. *BMC Med* (2008) 6:20. doi: 10.1186/1741-7015-6-20
208. Kanczuga-Koda L, Sulkowski S, Lenczewski A, Koda M, Wincewicz A, Baltaziak M, et al. Increased expression of connexins 26 and 43 in lymph node metastases of breast cancer. *J Clin Pathol* (2006) 59:429–33. doi: 10.1136/jcp.2005.029272
209. Pollmann MA, Shao Q, Laird DW, Sandig M. Connexin 43 mediated gap junctional communication enhances breast tumor cell diapedesis in culture. *Breast Cancer Res* (2005) 7:R522–34. doi: 10.1186/bcr1042
210. Villares GJ, Dobroff AS, Wang H, Zigler M, Melnikova VO, Huang L, et al. Overexpression of protease-activated receptor-1 contributes to melanoma metastasis via regulation of connexin 43. *Cancer Res* (2009) 69:6730–7. doi: 10.1158/0008-5472.CAN-09-0300
211. Bejarano E, Girao H, Yuste A, Patel B, Marques C, Spray DC, et al. Autophagy modulates dynamics of connexins at the plasma membrane in a ubiquitin-dependent manner. *Mol Biol Cell* (2012) 23:2156–69. doi: 10.1091/mbc.E11-10-0844
212. Bejarano E, Yuste A, Patel B, Stout RF Jr., Spray DC, Cuervo AM. Connexins modulate autophagosome biogenesis. *Nat Cell Biol* (2014) 16:401–14. doi: 10.1038/ncb2934
213. Lichtenstein A, Minogue PJ, Beyer EC, Berthoud VM. Autophagy: a pathway that contributes to connexin degradation. *J Cell Sci* (2011) 124:910–20. doi: 10.1242/jcs.073072
214. Iyyathurai J, Decuypere JP, Leybaert L, D'Hondt C, Bultynck G. Connexins: substrates and regulators of autophagy. *BMC Cell Biol* (2016) 17 Suppl 1:20. doi: 10.1186/s12860-016-0093-9
215. Elste AP, Petersen I. Expression of proteinase-activated receptor 1-4 (PAR 1-4) in human cancer. *J Mol Histol* (2010) 41:89–99. doi: 10.1007/s10735-010-9274-6
216. Wang M, An S, Wang D, Ji H, Geng M, Guo X, et al. Quantitative Proteomics Identify the Possible Tumor Suppressive Role of Protease-Activated Receptor-4 in Esophageal Squamous Cell Carcinoma Cells. *Pathol Oncol Res* (2019) 25:937–43. doi: 10.1007/s12253-018-0395-7
217. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* (2008) 105:10513–8. doi: 10.1073/pnas.0804549105
218. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res* (2008) 18:997–1006. doi: 10.1038/cr.2008.282
219. Liang L, Fu J, Wang S, Cen H, Zhang L, Mandukhail SR, et al. MiR-142-3p enhances chemosensitivity of breast cancer cells and inhibits autophagy by targeting HMGB1. *Acta Pharm Sin B* (2020) 10:1036–46. doi: 10.1016/j.apsb.2019.11.009
220. Zhou DM, Ran F, Ni HZ, Sun LL, Xiao L, Li XQ, et al. Metformin inhibits high glucose-induced smooth muscle cell proliferation and migration. *Aging (Albany NY)* (2020) 12:5352–61. doi: 10.18632/aging.102955
221. Sun Y, Liu X, Zhang D, Liu F, Cheng Y, Ma Y, et al. Platelet-Derived Exosomes Affect the Proliferation and Migration of Human Umbilical Vein Endothelial Cells Via miR-126. *Curr Vasc Pharmacol* (2018) 17:379–87. doi: 10.2174/1570161116666180313142139
222. Li M, Meng X, Li M. MiR-126 promotes esophageal squamous cell carcinoma via inhibition of apoptosis and autophagy. *Aging (Albany NY)* (2020) 12:12107–18. doi: 10.18632/aging.103379
223. Nguyen DX, Bos PD, Massagué J. Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer* (2009) 9:274–84. doi: 10.1038/nrc2622
224. van Nieuw Amerongen GP, van Delft S, Vermeer MA, Collard JG, van Hinsbergh VW. Activation of RhoA by thrombin in endothelial hyperpermeability: role of Rho kinase and protein tyrosine kinases. *Circ Res* (2000) 87:335–40. doi: 10.1161/01.res.87.4.335
225. Leonard A, Millar MW, Slavin SA, Bijli KM, Dionisio Santos DA, Dean DA, et al. Critical role of autophagy regulator Beclin1 in endothelial cell inflammation and barrier disruption. *Cell Signal* (2019) 61:120–9. doi: 10.1016/j.cellsig.2019.04.013
226. Slavin SA, Leonard A, Grose V, Fazal F, Rahman A. Autophagy inhibitor 3-methyladenine protects against endothelial cell barrier dysfunction in acute lung injury. *Am J Physiol Lung Cell Mol Physiol* (2018) 314:L388–96. doi: 10.1152/ajplung.00555.2016
227. Chao CH, Chen HR, Chuang YC, Yeh TM. Macrophage Migration Inhibitory Factor-Induced Autophagy Contributes to Thrombin-Triggered Endothelial Hyperpermeability in Sepsis. *Shock* (2018) 50:103–11. doi: 10.1097/SHK.0000000000000976
228. Bair AM, Thippegowda PB, Freichel M, Cheng N, Ye RD, Vogel SM, et al. Ca 2+ entry via TRPC channels is necessary for Thrombin-induced NF-kB activation in endothelial cells through AMP-activated protein kinase and protein kinase Cδ. *J Biol Chem* (2009) 284:563–74. doi: 10.1074/jbc.M803984200
229. Tiruppathi C, Naqvi T, Sandoval R, Mehta D, Malik AB. Synergistic effects of tumor necrosis factor-alpha and thrombin in increasing endothelial permeability. *Am J Physiol Lung Cell Mol Physiol* (2001) 281:L958–68. doi: 10.1152/ajplung.2001.281.4.L958
230. Stahmann N, Woods A, Carling D, Heller R. Thrombin Activates AMP-Activated Protein Kinase in Endothelial Cells via a Pathway Involving Ca2 +/Calmodulin-Dependent Protein Kinase Kinase β. *Mol Cell Biol* (2006) 26:5933–45. doi: 10.1128/mcb.00383-06
231. Thors B, Halldórsson H, Thorgeirsson G. ENOS activation mediated by AMPK after stimulation of endothelial cells with histamine or thrombin is dependent on LKB1. *Biochim Biophys Acta - Mol Cell Res* (2011) 1813:322–31. doi: 10.1016/j.bbamcr.2010.12.001
232. Wu Q, Hu Y, Jiang M, Wang F, Gong G. Effect of autophagy regulated by sirt1/FoxO1 pathway on the release of factors promoting thrombosis from vascular endothelial cells. *Int J Mol Sci* (2019) 20:1–14. doi: 10.3390/ijms20174132

233. Narita M, Young ARJ, Arakawa S, Samarajiwa SA, Nakashima T, Yoshida S, et al. Spatial coupling of mTOR and autophagy augments secretory phenotypes. *Science* (2011) 332:966–70. doi: 10.1126/science.1205407
234. Kawecki C, Lenting PJ, Denis CV. von Willebrand factor and inflammation. *J Thromb Haemost* (2017) 15:1285–94. doi: 10.1111/jth.13696
235. Rautou PE, Busse J, Kheloufi M, Vion AC, Boulaftali Y, Stark K, et al. Endothelial Autophagy Does Not Influence Venous Thrombosis in Mice. *Thromb Haemost* (2018) 118:1113–5. doi: 10.1055/s-0038-1641753
236. Sung BH, Ketova T, Hoshino D, Zijlstra A, Weaver AM. Directional cell movement through tissues is controlled by exosome secretion. *Nat Commun* (2015) 6:7164. doi: 10.1038/ncomms8164
237. Kerk N, Strozyk EA, Pöppelmann B, Schneider SW. The mechanism of melanoma-associated thrombin activity and von Willebrand factor release from endothelial cells. *J Invest Dermatol* (2010) 130:2259–68. doi: 10.1038/jid.2010.136
238. Xu Y, Pan S, Liu J, Dong F, Cheng Z, Zhang J, et al. GATA3-induced vWF upregulation in the lung adenocarcinoma vasculature. *Oncotarget* (2017) 8:110517–29. doi: 10.18632/oncotarget.22806
239. Patmore S, Dhami SPS, O'Sullivan JM. Von Willebrand Factor and Cancer; metastasis and coagulopathies. *J Thromb Haemost*. (2020). doi: 10.1111/jth.14976.
240. Ling J, Sun Y, Pan J, Wang H, Ma Z, Yin J, et al. Feedback modulation of endothelial cells promotes epithelial-mesenchymal transition and metastasis of osteosarcoma cells by Von Willebrand Factor release. *J Cell Biochem* (2019) 120:15971–9. doi: 10.1002/jcb.28875
241. Gomes N, Legrand C, Fauvel-Lafève F. Shear stress induced release of von Willebrand factor and thrombospondin-1 in HUVEC extracellular matrix enhances breast tumour cell adhesion. *Clin Exp Metastasis* (2005) 22:215–23. doi: 10.1007/s10585-005-7359-5
242. Morganti M, Carpi A, Amo-Takyi B, Sagripanti A, Nicolini A, Giardino R, et al. Von Willebrand's factor mediates the adherence of human tumoral cells to human endothelial cells and ticlopidine interferes with this effect. *BioMed Pharmacother* (2000) 54:431–6. doi: 10.1016/s0753-3322(00)00006-8
243. O'Sullivan JM, Preston RJS, Robson T, O'Donnell JS. Emerging Roles for von Willebrand Factor in Cancer Cell Biology. *Semin Thromb Hemost* (2018) 44:159–66. doi: 10.1055/s-0037-1607352
244. Grimsey NJ, Coronel LJ, Cordova IC, Trejo J. Recycling and endosomal sorting of protease-activated receptor-1 is distinctly regulated by Rab11A and Rab11B proteins. *J Biol Chem* (2016) 291:2223–36. doi: 10.1074/jbc.M115.702993
245. Martinez-Outschoorn UE, Whitaker-Menezes D, Lin Z, Flomenberg N, Howell A, Pestell RG, et al. Cytokine production and inflammation drive autophagy in the tumor microenvironment: Role of stromal caveolin-1 as a key regulator. *Cell Cycle* (2011) 10:1784–93. doi: 10.4161/cc.10.11.15674
246. Wang R, Zhang T, MA Z, Wang Y, Cheng Z, Xu H, et al. The interaction of coagulation factor XII and monocyte/macrophages mediating peritoneal metastasis of epithelial ovarian cancer. *Gynecol Oncol* (2010) 117:460–6. doi: 10.1016/j.ygyno.2010.02.015
247. Renné T, Stavrou EX. Roles of Factor XII in Innate Immunity. *Front Immunol* (2019) 10:2011. doi: 10.3389/fimmu.2019.02011
248. Ma Z, Zhang T, Wang R, Cheng Z, Xu H, Li W, et al. Tissue factor-factor VIIa complex induces epithelial ovarian cancer cell invasion and metastasis through a monocytes-dependent mechanism. *Int J Gynecol Cancer* (2011) 21:616–24. doi: 10.1097/IGC.0b013e3182150e98
249. Ma YY, He XJ, Wang HJ, Xia YJ, Wang SL, Ye ZY, et al. Interaction of coagulation factors and tumor-associated macrophages mediates migration and invasion of gastric cancer. *Cancer Sci* (2011) 102:336–42. doi: 10.1111/j.1349-7006.2010.01795.x
250. Lindholm PF, Lu Y, Adley BP, Vladislav T, Jovanovic B, Sivapurapu N, et al. Role of monocyte-lineage cells in prostate cancer cell invasion and tissue factor expression. *Prostate* (2010) 70:1672–82. doi: 10.1002/pros.21202
251. Deng C, Wu S, Zhang L, Yang M, Lin Q, Xie Q, et al. Role of monocyte tissue factor on patients with non-small cell lung cancer. *Clin Respir J* (2018) 12 (3):1125–33. doi: 10.1111/crj.12640
252. Antoniak S, MacKman N. Multiple roles of the coagulation protease cascade during virus infection. *Blood* (2014) 123:2605–13. doi: 10.1182/blood-2013-09-526277
253. Vergadi E, Ieronymaki E, Lyroni K, Vaporidi K, Tsatsanis C. Akt Signaling Pathway in Macrophage Activation and M1/M2 Polarization. *J Immunol* (2017) 198:1006–14. doi: 10.4049/jimmunol.1601515
254. Haloul M, Oliveira ERA, Kader M, Wells JZ, Tominello TR, El Andaloussi A, et al. mTORC1-mediated polarization of M1 macrophages and their accumulation in the liver correlate with immunopathology in fatal ehrlichiosis. *Sci Rep* (2019) 9:14050. doi: 10.1038/s41598-019-50320-y
255. Xu Y, Liu X, Gong X, Eissa NT. Signaling pathway of autophagy associated with innate immunity. *Autophagy* (2008) 4:1:110–2. doi: 10.4161/auto.5225
256. Liao X, Sluimer JC, Wang Y, Subramanian M, Brown K, Pattison JS, et al. Macrophage autophagy plays a protective role in advanced atherosclerosis. *Cell Metab* (2012) 15(4):545–553. doi: 10.1016/j.cmet.2012.01.022
257. Mihalache CC, Simon HU. Autophagy regulation in macrophages and neutrophils. *Exp Cell Res* (2012) 318(11):1187–92. doi: 10.1016/j.yexcr.2011.12.021
258. Wu M-Y, Lu J-H. Autophagy and Macrophage Functions: Inflammatory Response and Phagocytosis. *Cells* (2019) 9(1):70. doi: 10.3390/cells9010070
259. Massberg S, Grahl L, von Bruehl ML, Manukyan D, Pfeiler S, Goosmann C. Reciprocal coupling of coagulation and innate immunity via neutrophil serine proteases. *Nat Med* 16(8):887–96. doi: 10.1038/nm.2184
260. Jung HS, Gu J, Kim JE, Nam Y, Song JW, Kim HKCancer cell-induced neutrophil extracellular traps promote both hypercoagulability and cancer progression. *PLoS One* (2019) 14(4):e0216055. doi: 10.1371/journal.pone.0216055
261. Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers JDD, et al. Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci U S A* (2010) 107:15880–5. doi: 10.1073/pnas.1005743107
262. Maugeri N, Campana L, Gavina M, Covino C, De Metrio M, Panciroli C, et al. Activated platelets present high mobility group box 1 to neutrophils, inducing autophagy and promoting the extrusion of neutrophil extracellular traps. *J Thromb Haemost* (2014) 12(12):2074–88. doi: 10.1111/jth.12710
263. McDonald B, Davis RP, Kim SJ, Tse M, Esmon CT, Kolaczkowska E, et al. Platelets and neutrophil extracellular traps collaborate to promote intravascular coagulation during sepsis in mice. *Blood* (2017) 129:1357–67. doi: 10.1182/blood-2016-09-741298
264. Cedervall J, Zhang Y, Olsson AK. Tumor-Induced NETosis as a Risk Factor for Metastasis and Organ Failure. *Cancer Res* (2016) 76:4311–5. doi: 10.1158/0008-5472.CAN-15-3051
265. Jorch SK, Kubes P. An emerging role for neutrophil extracellular traps in noninfectious disease. *Nat Med* (2017) 23:279–87. doi: 10.1038/nm.4294
266. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. *Nat Rev Immunol* (2018) 18:134–47. doi: 10.1038/nri.2017.105
267. Olsson AK, Cedervall J. NETosis in Cancer - Platelet-Neutrophil Crosstalk Promotes Tumor-Associated Pathology. *Front Immunol* (2016) 7:373. doi: 10.3389/fimmu.2016.00373
268. Liu K, Zhao E, Ilyas G, Lalazar G, Lin Y, Haseeb M, et al. Impaired macrophage autophagy increases the immune response in obese mice by promoting proinflammatory macrophage polarization. *Autophagy* (2015) 11:271–84. doi: 10.1080/15548627.2015.1009787
269. Mihara K, Ramachandran R, Renaux B, Saifeddine M, Hollenberg MD. Neutrophil elastase and proteinase-3 trigger G proteinbiased signaling through proteinase-activated receptor-1 (PAR1). *J Biol Chem* (2013) 288:32979–90. doi: 10.1074/jbc.M113.483123
270. Kambas K, Mitroulis I, Apostolidou E, Girod A, Chrysanthopoulou A, Pneumatikos I, et al. Autophagy Mediates the Delivery of Thrombogenic Tissue Factor to Neutrophil Extracellular Traps in Human Sepsis. *PloS One* (2012) 7:1–14. doi: 10.1371/journal.pone.0045427
271. Boone BA, Murthy P, Miller-Ocuin J, Doerfler WR, Ellis JT, Liang X, et al. Chloroquine reduces hypercoagulability in pancreatic cancer through inhibition of neutrophil extracellular traps. *BMC Cancer* (2018) 18:1–12. doi: 10.1186/s12885-018-4584-2
272. Demers M, Krause DS, Schatzberg D, Martinod K, Voorhees JR, Fuchs TA, et al. Cancers predispose neutrophils to release extracellular DNA traps that contribute to cancer-associated thrombosis. *Proc Natl Acad Sci U S A* (2012) 109(32):13076–81. doi: 10.1073/pnas.1200419109
273. Boone BA, Schapiro NE, Neal MD, Ellis JT, Lotze MT, Zeh HJ. Neutrophil Extracellular Traps (NETs) are Upregulated in Pancreatic Cancer as a Result of Autophagy and Promote Hypercoagulability. *J Surg Res* (2014) 186(2):638. doi: 10.1016/j.jss.2013.11.657
274. Boone BA, Orlichenko L, Schapiro NE, Loughran P, Gianfrate GC, Ellis JT, et al. The receptor for advanced glycation end products (RAGE) enhances

autophagy and neutrophil extracellular traps in pancreatic cancer. *Cancer Gene Ther* (2015) 22:326–34. doi: 10.1038/cgt.2015.21
275. Maugeri N, Capobianco A, Rovere-Querini P, Ramirez GA, Tombetti E, Della Valle P, et al. Platelet microparticles sustain autophagy-associated activation of neutrophils in systemic sclerosis. *Sci Transl Med* (2018) 10: eaao3089. doi: 10.1126/scitranslmed.aao3089
276. Young A, Chapman O, Connor C, Poole C, Rose P, Kakkar AK. Thrombosis and cancer. *Nat Rev Clin Oncol* (2012) 9(8):437–49. doi: 10.1038/nrclinonc.2012.106
277. Alexander ET, Minton AR, Peters MC, van Ryn J, Gilmour SK. Thrombin inhibition and cisplatin block tumor progression in ovarian cancer by alleviating the immunosuppressive microenvironment. *Oncotarget* (2016) 7 (51):85291–305. doi: 10.18632/oncotarget.13300
278. Covic L, Kuliopulos A. Protease-activated receptor 1 as therapeutic target in breast, lung, and ovarian cancer: Pepducin approach. *Int J Mol Sci* (2018) 19 (8):2237. doi: 10.3390/ijms19082237
279. Versteeg HH, Schaffner F, Kerver M, Petersen HH, Ahamed J, Felding-Habermann B, et al. Inhibition of tissue factor signaling suppresses tumor growth. *Blood* (2008) 111:190–9. doi: 10.1182/blood-2007-07-101048
280. Versteeg HH, Ruf W. Emerging insights in tissue factor-dependent signaling events. *Semin Thromb Hemost* (2006) 32:24–32. doi: 10.1055/s-2006-933337
281. Cho J, Furie BC, Coughlin SR, Furie B. A critical role for extracellular protein disulfide isomerase during thrombus formation in mice. *J Clin Invest* (2008) 118(3):1123–31. doi: 10.1172/JCI34134
282. Wendel HG, De Stanchina E, Fridman JS, Malina A, Ray S, Kogan S, et al. Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. *Nature* (2004) 428(6980):332–7. doi: 10.1038/nature02369
283. Mack JM, Verkamp B, Richter GT, Nicholas R, Stewart K, Crary SE. Effect of sirolimus on coagulopathy of slow-flow vascular malformations. *Pediatr Blood Cancer* (2019) 66(10):e27896. doi: 10.1002/pbc.27896
284. Liu Z, Yan Y, Cao S, Chen Y. Long non-coding RNA SNHG14 contributes to gastric cancer development through targeting miR-145/SOX9 axis. *J Cell Biochem* (2018) 119:6905–13. doi: 10.1002/jcb.26889
285. Guba M, Yezhelyev M, Eichhorn ME, Schmid G, Ischenko I, Papyan A, et al. Rapamycin induces tumor-specific thrombosis via tissue factor in the presence of VEGF. *Blood* (2005) 105(11):4463–9. doi: 10.1182/blood-2004-09-3540
286. Tanaka H, Hino H, Moriya S, Kazama H, Miyazaki M, Takano N, et al. Comparison of autophagy inducibility in various tyrosine kinase inhibitors and their enhanced cytotoxicity via inhibition of autophagy in cancer cells in combined treatment with azithromycin. *Biochem Biophys Rep* (2020) 22:100750. doi: 10.1016/j.bbrep.2020.100750
287. Mukai S, Moriya S, Hiramoto M, Kazama H, Kokuba H, Che XF, et al. Macrolides sensitize EGFR-TKI-induced non-apoptotic cell death via blocking autophagy flux in pancreatic cancer cell lines. *Int J Oncol* (2016). doi: 10.3892/ijo.2015.3237
288. Zhao L, Yang G, Shi Y, Su C, Chang J. Co-delivery of Gefitinib and chloroquine by chitosan nanoparticles for overcoming the drug acquired resistance. *J Nanobiotechnology* (2015) 13:57. doi: 10.1186/s12951-015-0121-5
289. Liu Z, He K, Ma Q, Yu Q, Liu C, Ndege I, et al. Autophagy inhibitor facilitates gefitinib sensitivity in vitro and in vivo by activating mitochondrial apoptosis in triple negative breast cancer. *PloS One* (2017) 12(5):e0177694. doi: 10.1371/journal.pone.0177694
290. Strauss L, Mahmoud MAA, Weaver JD, Tijaro-Ovalle NM, Christofides A, Wang Q, et al. Targeted deletion of PD-1 in myeloid cells induces antitumor immunity. *Sci Immunol* (2020) 5(43):eaay1863. doi: 10.1126/sciimmunol.aay1863

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Hill, Hernández-Cáceres, Asencio, Torres, Solis and Owen. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Oncology

REVIEW
published: 09 December 2020
doi: 10.3389/fonc.2020.602661

Role of Autophagy in the Microenvironment of Oral Squamous Cell Carcinoma

Daniel Peña-Oyarzún[1,2,3,4], Montserrat Reyes[5], María Paz Hernández-Cáceres[3,4], Catalina Kretschmar[1,2,3], Eugenia Morselli[3,4], Cesar A. Ramirez-Sarmiento[6], Sergio Lavandero[1,7], Vicente A. Torres[1,2] and Alfredo Criollo[1,2,3*]*

[1] Advanced Center for Chronic Disease (ACCDiS), Facultad de Ciencias Químicas & Farmacéuticas and Facultad de Medicina, Universidad de Chile, Santiago, Chile, [2] Facultad de Odontología, Instituto de Investigación en Ciencias Odontológicas, Universidad de Chile, Santiago, Chile, [3] Autophagy Research Center, Universidad de Chile, Santiago, Chile, [4] Departamento de Fisiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile, [5] Departamento de Patología y Medicina Oral, Facultad de Odontología, Universidad de Chile, Santiago, Chile, [6] Facultades de Ingeniería, Medicina y Ciencias Biológicas, Institute for Biological and Medical Engineering, Pontificia Universidad Católica de Chile, Santiago, Chile, [7] Cardiology Division, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX, United States

OPEN ACCESS

Edited by:
Laura Pellegrini, Institute of Oncology Research (IOR), Switzerland

Reviewed by:
Hisashi Harada, Virginia Commonwealth University, United States
Martin R. Berger, German Cancer Research Center (DKFZ), Germany

***Correspondence:**
Vicente A. Torres
vatorres@odontologia.uchile.cl
Alfredo Criollo
alcriollo@u.uchile.cl

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *04 September 2020*
Accepted: *10 November 2020*
Published: *09 December 2020*

Citation:
Peña-Oyarzún D, Reyes M, Hernández-Cáceres MP, Kretschmar C, Morselli E, Ramirez-Sarmiento CA, Lavandero S, Torres VA and Criollo A (2020) Role of Autophagy in the Microenvironment of Oral Squamous Cell Carcinoma. Front. Oncol. 10:602661. doi: 10.3389/fonc.2020.602661

Oral squamous cell carcinoma, the most common type of oral cancer, affects more than 275,000 people per year worldwide. Oral squamous cell carcinoma is very aggressive, as most patients die after 3 to 5 years post-diagnosis. The initiation and progression of oral squamous cell carcinoma are multifactorial: smoking, alcohol consumption, and human papilloma virus infection are among the causes that promote its development. Although oral squamous cell carcinoma involves abnormal growth and migration of oral epithelial cells, other cell types such as fibroblasts and immune cells form the carcinoma niche. An underlying inflammatory state within the oral tissue promotes differential stress-related responses that favor oral squamous cell carcinoma. Autophagy is an intracellular degradation process that allows cancer cells to survive under stress conditions. Autophagy degrades cellular components by sequestering them in vesicles called autophagosomes, which ultimately fuse with lysosomes. Although several autophagy markers have been associated with oral squamous cell carcinoma, it remains unclear whether up- or down-regulation of autophagy favors its progression. Autophagy levels during oral squamous cell carcinoma are both timing- and cell-specific. Here we discuss how autophagy is required to establish a new cellular microenvironment in oral squamous cell carcinoma and how autophagy drives the phenotypic change of oral squamous cell carcinoma cells by promoting crosstalk between carcinoma cells, fibroblasts, and immune cells.

Keywords: oral squamous cell carcinoma, autophagy, tumor microenvironment, cancer, carcinoma-associated fibroblast

INTRODUCTION

The study of the tumor microenvironment has gained attention during the last decade and the development of effective anti-cancer therapies has been challenging (1). Tumors are not just masses of growing cells, but a novel tissue with evolving features over time (2). Tumors contain, besides primary tumor cells, stromal cells (including fibroblasts, vascular endothelial cells and adipocytes, among others) and immune cells (such as macrophages, T cells and B cells), which assist tumor initiation and/or progression (2).

Oral squamous cell carcinoma (OSCC) is the most common type of oral cancer and current treatments are limited to surgery, radiotherapy, chemotherapy or a combination of these, inflicting a huge impact on the quality of life of the patients, such as speech impairment, swallowing difficulties and face remodeling (3). Understanding the cellular mechanisms that allow communication between primary tumor cells and the tumor microenvironment can be crucial to find novel pharmacological targets for the treatment of OSCC. One of these mechanisms is autophagy, an intracellular degradation process that behaves as a "double-edge sword" when it comes to cancer (4). Treatment of different types of cancer, including OSCC, with autophagy modulators yields either promising or devastating results (5). Here we summarize and discuss the evidence regarding the role of autophagy in the communication of OSCC cells and their microenvironment. This review will hopefully shed some light on the contradictory results of autophagy-based treatments in OSCC.

ORAL SQUAMOUS CELL CARCINOMA

Oral cancer is one of the head and neck cancers which represent the sixth most common malignancy in the world (6, 7). Oral cancer affects nearly 300,000 persons each year, mainly in high-income countries (8, 9). Oral cancer is twice as common in men as in women and the average age for diagnosis is 62, although it also occurs in younger people (10). Importantly, only 40%–50% of patients have a 5-year survival, and if there is metastasis, the average 5-year survival rate is 39%. However, survival is 84% at 5 years if the diagnosis is made at an early stage. Therefore, early diagnosis of oral cancer is a decisive factor in improving patient survival (10).

About 90% of oral cancers originate in the stratified non-keratinized epithelium of the oral mucosa, which is the reason for its denomination as oral squamous cell carcinoma. Its main risk factors include consumption of tobacco and alcohol, along with other possible risk factors, such as chronic irritation, poor oral hygiene, human papillomavirus (HPV), malnutrition and immune system suppression (11–13). These risk factors provoke the development of various genetic instabilities and molecular alterations, including the loss of heterozygosity of chromosomes 3, 4 7, 8, 11, 17, and 19, among others, down-regulation of tumor-suppressor genes such as TP53, RB, CDKN2A, and up-regulation of oncogenes such as cyclin D1 (6, 14, 15). Following exposure to the carcinogens mentioned above, normal oral keratinocytes form an epithelial dysplasia, which is a tissue alteration where cells adapt to stressful stimuli by changing their number and shape. Continuous exposure to carcinogens shifts the progression of the epithelial dysplasia from mild to severe, ending with its malignant transformation to OSCC and metastasis (16). Metastasis of OSCC cells is mainly through the lymphatic vessels to the cervical lymph nodes on the same side of the face, which plays a critical role in the management and prognosis of patients with OSCC. The most common distant metastatic sites are the lungs, liver and bones (17, 18). The pathological progression, as well as the global burden and survival chances after diagnosis of OSCC, are depicted in **Figure 1**.

The clinical presentation of OSCC is highly variable; the most common is observed as an ulcerated lesion in the oral cavity that does not heal, containing harsh edges by palpation, while other signs may include mobile teeth, bleeding, pain or numbness in the mouth or face (9, 10). Treatment options are limited to surgical resection as a primary treatment, and radiation as a primary treatment or as an adjuvant after surgery. Chemotherapy is used mainly as adjuvant after surgery, given that pharmacological treatments usually have secondary effects (19). In most cases surgery, radiation and chemotherapy lead to negative effects on the patient's quality of life, such as speaking impairment, swallowing dysfunction, physical appearance alteration, sensory disability and chronic pain (19, 20). Thus a deeper understanding of the cellular and molecular biology of OSCC, regarding its development and progression, is required to improve pharmacological treatments and avoid secondary effects.

In this context, it has been acknowledged that the tumor microenvironment plays a vital role in OSCC progression and invasion, as it directly affects both tumor growth and its ability to progress and metastasize. Here, blood vessels, nerves and immune cells contribute to the tumor heterogeneity limiting therapeutic access, altering drug metabolism and contributing to drug resistance (21).

THE MICROENVIRONMENT OF THE OSCC

Stromal cancer-associated fibroblasts, CAFs, are the primary non-immune infiltrative cells in the carcinoma microenvironment

Abbreviations: α-SMA, alpha smooth muscle actin; AMPK, AMP-activated protein kinase; ATG, autophagy-related protein; BECN1, Beclin 1; CAF, cancer-associated fibroblast; CCL, chemokine (C-C motif) ligand; CCR, chemokine (C-C motif) receptors; CXCL, chemokine (C-X-C motif) ligand; CD, cluster of differentiation; DLC-1, Deleted in Liver Cancer; EGF, epithelial growth factor; EMT, epithelial-mesenchymal transition; FAK, focal adhesion kinase; HSP90, heat shock protein 90; HPV, human papillomavirus; HIF1α, hypoxia inducible factor 1 subunit alpha; IκB, inhibitor of NFκB; IL, interleukin; IL1β, interleukin-1β; JAK2, Janus kinase 2; lncRNA, long non-coding RNA; mTOR, mechanistic target of rapamycin; MMP, metalloproteinase; miR, microRNA; MAP1LC3/LC3, Microtubule Associated Protein 1 Light Chain 3; MCP-1, monocyte chemoattractant protein-1; MDX1, Myc-competitor MAD; NFκB, nuclear factor κB; OSCC, oral squamous cell carcinoma; PtdIns3K, Phosphatidylinositol 3-phosphate kinase complex; PFK, phosphofructokinase; PDGF-Rβ, platelet-derived growth factor receptor β; RANKL, receptor activator of nuclear factor κB ligand; ROCK2, Rho-associated coiled-coil kinase 2; SQSTM1/p62, Sequestrosome 1; STAT3, signal transducer and activator of transcription protein 3; TLR4, Toll-like receptor 4; TGFβ, transforming growth factor β; TAM, tumor-associated macrophage; TAN, tumor-associated neutrophil; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; ULK1, Unc-51-like autophagy activating kinase; VEGF, vascular endothelial growth factor.

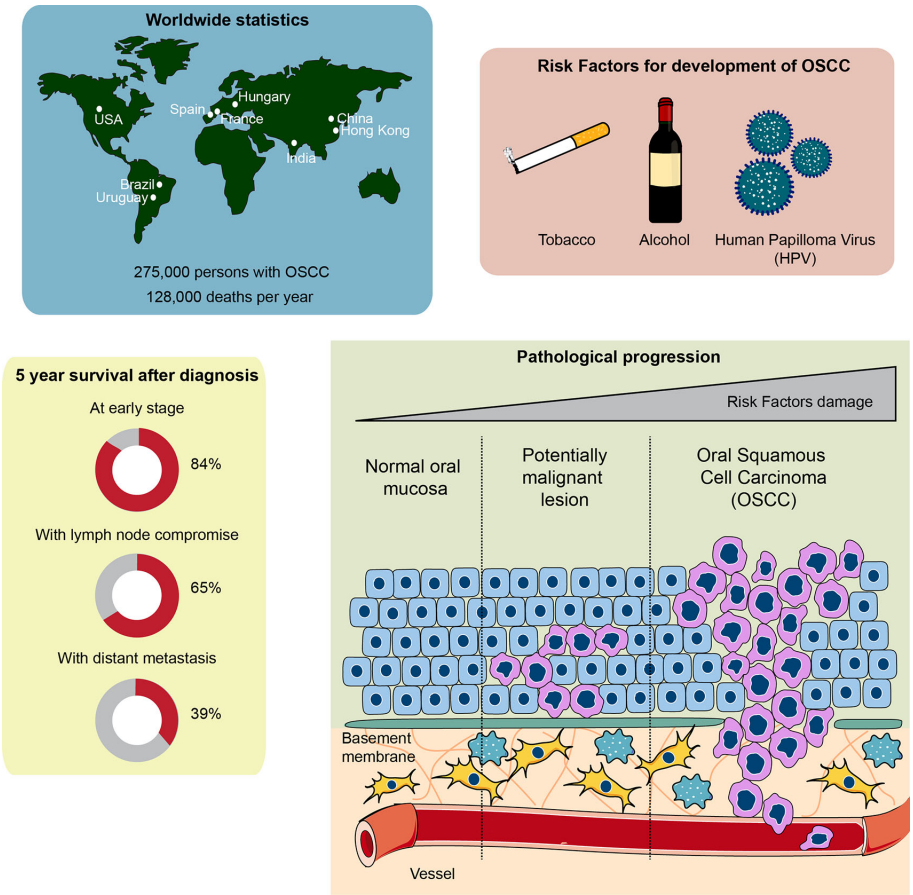


FIGURE 1 | Global statistics, survival rate, and pathophysiological features of oral squamous cell carcinoma (OSCC). Top left: countries with higher cases of OSCC diagnosed around the world. Top right: the main risk factors involved in OSCC development and progression. Bottom left: chances of survival 5 years after being diagnosed with OSCC. Note that early diagnosis of OSCC is crucial to ensure over 80% survival chance after 5 years. The general statistics show a 50% survival rate after 5 years, given that OSCC is usually diagnosed late. Bottom right: development of OSCC from a normal oral epithelium. The normal epithelium, composed of epithelial cells known as keratinocytes, is located over a basement membrane that separates the epithelium from the connective tissue composed of fibroblasts, immune cells and vessels. Exposure to carcinogens derived from the risk factors of the top right panel generate a potentially malignant lesion, characterized by an altered cellular morphology that starts affecting the inner layers of the epithelium close to the basement membrane, progressing toward the outer layers of the epithelium. Continuous exposure to carcinogens leads to OSCC development, a phenomenon that alters all the epithelial cell layers both genetically and morphologically. Interplay between connective tissue cells and OSCC cells is also observed, which assists OSCC growth and metastasis.

(22). Studies have revealed that CAFs show increased expression of proteins involved in actin cytoskeleton remodeling during migration, such as Rho-associated coiled-coil kinase 2, ROCK2 (23), focal adhesion kinase, FAK (24), and alpha smooth muscle actin, α-SMA (25). *In vitro* studies reported that CAFs show higher migration rates compared to fibroblasts obtained from normal subjects (26), suggesting that events linked to the epithelial-mesenchymal transition (EMT) in CAFs may participate during OSCC progression. EMT of CAFs is related to increased levels of platelet-derived growth factor receptor β, PDGF-Rβ, in the plasma membrane (27), which in turn activates Janus kinase 2, JAK2, and the signal transducer and activator of transcription protein 3, STAT3 (25). Activation of the JAK2/STAT3 pathway in CAFs provokes the release of epidermal growth factor, EGF, which promotes the EMT in tumor cells (25). It has also been reported that CAFs release other factors that contribute to the EMT in tumor epithelial cells. Among these, CAFs generate exosomes containing microRNAs (miRs) such as miR-382-5p (28), which is associated with advanced TNM stages of the OSCC (29). Although the molecular mechanism by which miR-382-5p affects OSCC has not been totally elucidated, studies have shown that miR-382-5p is required to down-regulate the expression of the Myc-competitor MAD (MDX1) in breast cancer (30), as well as the expression of the negative regulator of cell motility Deleted in Liver Cancer, DLC-1, in hepatic cancer (31), suggesting that miR-382-5p may reduce the expression of tumor suppressor genes in OSCC. However, it is worth noting that OSCC-related CAFs may also reduce the delivery of specific miRs such as miR-34a-5p, which has been shown to reduce the expression of the tyrosine kinase receptor AXL, decreasing β-catenin-dependent proliferation and SNAIL-

dependent expression of metalloproteinases 2 (MMP2) and 9 (MMP9) (32). This reveals that OSCC-related CAFs selectively promote the release of pro-tumoral miRs over anti-tumoral miRs.

OSCC cells promote the release of several chemokines from CAFs, leading either to immune infiltration or changes in OSCC phenotype toward a pro-migratory and proliferative phenotype. For instance, OSCC cells release interleukin-1β, IL1β, which in turn provokes the release of the chemokine (C-C motif) ligand 7, CCL7, from CAFs (33). Then CCL7 binds to the chemokine (C-C motif) receptors 1-3, CCR1-3, located in the OSCC cells, increasing cell migration *in vitro* (33). The chemokine CCL2, also known as monocyte chemoattractant protein-1, MCP-1, is released by CAFs, being positively associated with lymph node metastasis (24). CCL2 positive CAFs are observed at the lymphoid metastatic focus, specifically at the marginal sinus of OSCC (34). The activation of NFκB and STAT3, as a result of hypoxia in the tumor niche, can also induce expression and release of CCL2 from CAFs (35). On the other hand, hypoxia has been shown to promote the expression of galectin-1, a protein involved in FAK activation and migration (36). Notably, galectin-1 is required for CCL2 expression in CAFs, promoting OSCC tumor growth and intravasation in xenograft models (37).

The transformation of normal fibroblasts into CAFs is also mediated by molecules that are released from OSCC cells. For instance, IL1β expression becomes progressively increased in OSCC cells and is released, activating the NFκB pathway in fibroblasts that induces release of the chemokine (C-X-C motif) ligand 1, CXCL1 (38). CXCL1 generates an autocrine mechanism that transforms fibroblasts into high-α-SMA expressing CAFs (39), suggesting that early carcinogenesis events provoke slight inflammatory alterations in the epithelial cells that then lead to the generation of CAFs.

Infiltration of immune cells is observed in OSCC, mainly promoted by the cytokines released from CAFs. Tumor-associated neutrophils, TANs, and tumor-associated macrophages, TAMs, have been observed both at the primary tumor site and at the lymphoid metastatic focus (34). Studies have shown that anti-inflammatory mediators such as TGFβ and IL10 are released from CAFs, which prevent proliferation of T-cells and promote infiltration of CD163-positive TAMs (40, 41). The CD163 membrane marker of M2 macrophages is expressed during resolution of inflammation, indicating that infiltration of specialized immune cell triggers an immunosuppressive environment (42). Thus M2 macrophage infiltration may promote tumor angiogenesis and metastasis through the release of vascular endothelial growth factor, VEGF, and PDGF (43). The presence of CD163 TAMs correlates with lymph node invasion and poor prognosis of patients with OSCC (40, 44). TAMs may also induce OSCC cells proliferation *via* the release of EGF (45). Interestingly, both CAFs and OSCC cells depict reduced TGFβ receptors 2 and 3, suggesting that the cytostatic effect of TGFβ only affects immune cells (46, 47). Finally, CAFs also attract regulatory T-cells, T-regs, shutting down the inflammatory response of T-cells and sustaining the immunosuppressive environment of the tumor (48). As in the recruitment of TAMs, the attraction of T-regs to the OSCC primary site is mediated by TGFβ and IL10 (49).

In contrast to the immunosuppressive role of CAFs, most molecules released from OSCC cells, such as IL8 and IL6, depict pro-inflammatory effects as a result of the stressful insults (i.e. tobacco smoking) (50–52). Similar to the anti-inflammatory molecules released from CAFs, IL8 and IL6 are produced in an NFκB-dependent manner, suggesting that inhibition of the NFκB pathway may be suitable for the treatment of OSCC (50, 52). The contrasting inflammatory behavior of OSCC cells and CAFs may provide an alternative approach for the treatment of OSCC, since CAF-independent growth of OSCC cells may not be sustainable in time because it would alert the defensive branch of the immune system. Further studies are required to elucidate this.

The OSCC microenvironment is subjected to both pro- and anti-inflammatory mediators over time. Initial epithelial insults result in increased tumor features with underlying inflammation. Pro-inflammatory cytokines released by OSCC cells provoke the development of CAFs from normal fibroblasts, thereby sustaining proliferation, migration and invasion of the OSCC cells. Also, CAFs cause the infiltration of immune cells with immunosuppressive behavior, further assisting during the metastatic process of OSCC toward lymph nodes. These antecedents are summarized in **Figure 2**.

AUTOPHAGY IN STRESS AND CANCER

Autophagy is a cellular process, conserved from yeasts to mammals, that promotes the degradation of wasted intracellular materials such as macromolecules and organelles, to maintain the cell homeostasis (53). A basal autophagic tone is present in all cells, but autophagy is up-regulated under certain stress stimuli to cope with the damage (54). For instance, oral gingival cells exposed to tobacco smoke, alcohol consumption or HPV increase autophagy as a protective mechanism (55). Autophagy occurs with the formation of double-membrane vesicles known as autophagosomes. Autophagosomes sequester the material that is targeted for degradation, and ultimately fuse with the lysosome to form the autolysosome (56). The lysosome contains hydrolytic enzymes and a low pH that allows degradation of the materials sequestered by the autophagosome (57).

The proteins that participate during autophagy are known as autophagy-related proteins or ATGs (56). ATG8, known as Microtubule Associated Protein 1 Light Chain 3, MAP1LC3 (or just LC3), is critical for the autophagy mechanism (58). When autophagy is induced, LC3 is cleaved by the protease ATG4, forming LC3-I, and then conjugated by the ATG5-ATG7 complex with the lipid phosphatidylethanolamine, forming LC3-II (59). Then LC3-II binds to the autophagosome membrane and promotes its elongation (58). Detection of LC3 positive vesicles and LC3-II levels are usually performed to evaluate autophagy (60). Of note, autophagy is not the mere formation of autophagosomes, but also degradation of target materials within lysosomes. This is known as "autophagy flux", which indicates the progression from sequestration to degradation of the cargo (61). Chemical compounds such as bafilomycin-A1 and chloroquine, which inhibit the fusion between autophagosomes and lysosomes, blunt autophagy, as observed by the accumulation of autophagosomes (60). This is why conclusions from LC3 data alone should be

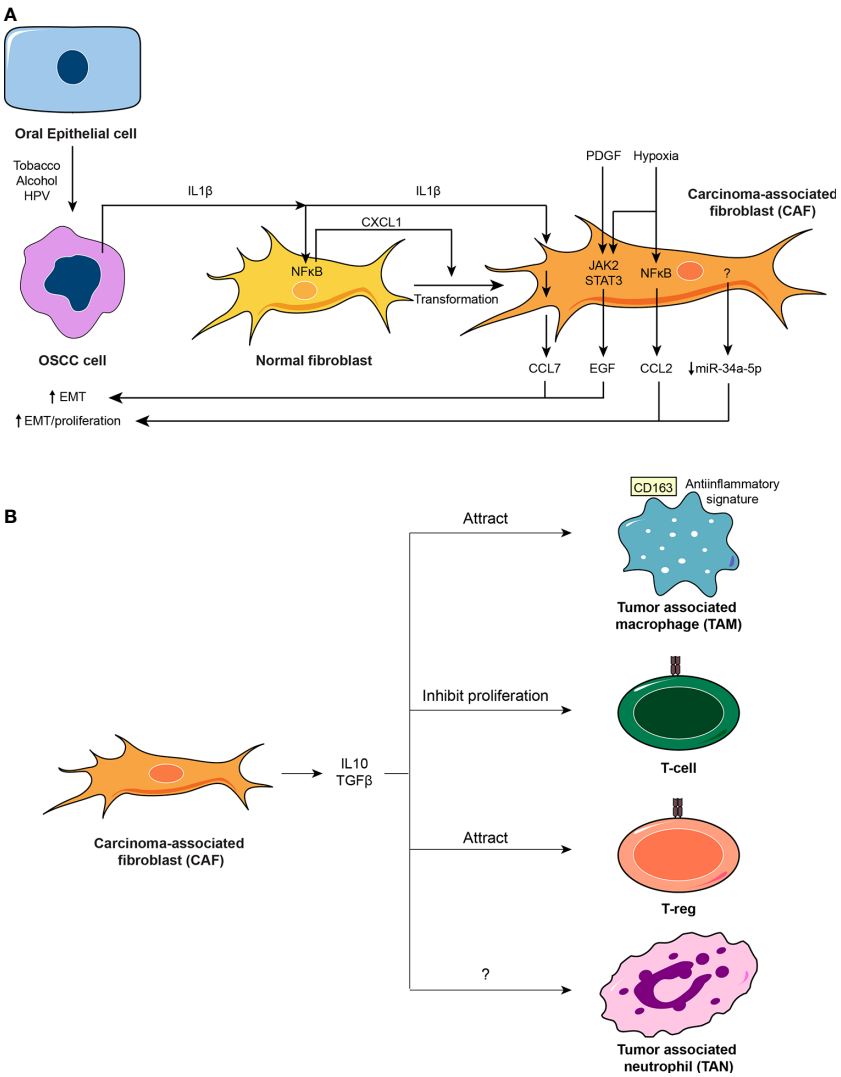


FIGURE 2 | The oral squamous cell carcinoma (OSCC) tumor microenvironment. **(A)** The OSCC tumor microenvironment is mainly composed of cancer-associated fibroblasts (CAFs). CAFs derive from normal fibroblasts after autocrine stimulation of chemokine (C-X-C motif) ligand (CXCL1) chemokine through a nuclear factor κB (NFκB)-dependent mechanism. NFκB is activated in fibroblasts by the action of interleukin-1β (IL1β) released from OSCC cells. Conditions surrounding the tumoral tissue, such as increased levels of platelet-derived growth factor (PDGF) and IL1β or hypoxia activate Janus kinase (JAK)/STAT and NFκB pathways in CAFs, which induce the release of chemokines (CCL2 and CCL7) and epithelial growth factor (EGF), or inhibit the release of selective miRs such as miR-34a-5p. All these mediators augment proliferation and EMT of OSCC cells. **(B)** CAFs release well known anti-inflammatory molecules such as IL10 and TGFβ that attract anti-inflammatory macrophages and regulatory T-cells, T-regs, and inhibit proliferation of T-cells.

managed with caution, as it will be discussed in the following section. Other markers besides LC3 are commonly determined to help to draw appropriate conclusions, such as Sequestrosome 1, SQSTM1 (also known as p62), which is a protein that binds poly-ubiquitinated proteins and LC3, carrying the proteins into the autophagosomes (62). SQSTM1/p62 is degraded along with the targets, leading to a reduction in its level (63). In contrast, autophagy flux blockage leads to increased levels of SQSTM1/p62.

Autophagy is controlled by stress signaling pathways that work as an on/off switch. This switch is known as ATG1 (also known as Unc-51-like autophagy activating kinase, ULK1), a kinase activated by AMP-activated protein kinase, AMPK, and inactivated by the mechanistic target of rapamycin, mTOR (64), a serine/threonine protein kinase. Both AMPK and mTOR are kinases that check the nutritional status of the cell. Under normal nutritional conditions, mTOR represses autophagy, while starvation increases AMPK activation and autophagy (65, 66). Thus, starvation and mTOR repression are common autophagy inducers. Active ATG1 phosphorylates and activates Beclin 1 (BECN1), a protein required to transform intracellular membranes into autophagic membranes (67). BECN1 assembles the phosphatidylinositol 3-phosphate kinase complex, PtdIns3K, which catalyzes the formation of phosphatidylinositol 3-phosphate from phosphatidylinositol, serving as an intracellular domain that recruits other ATG

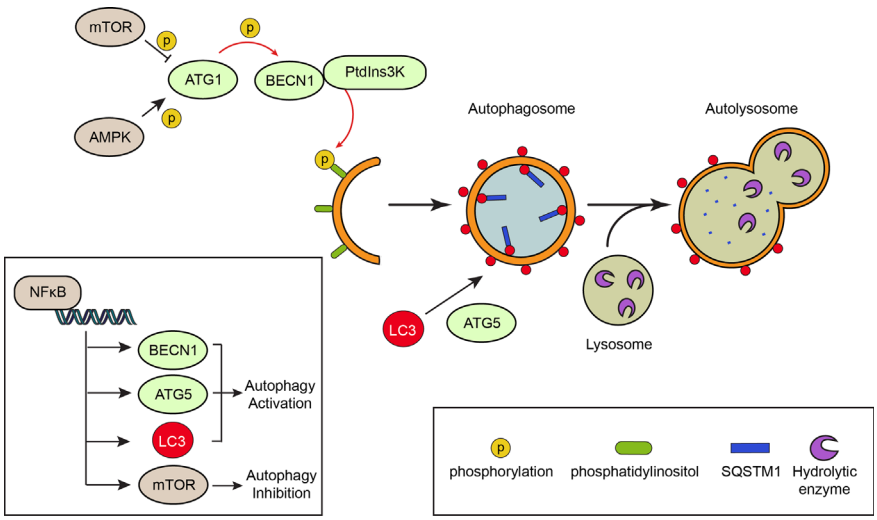


FIGURE 3 | The mechanism of autophagy. Autophagy is a degradation process that involves the formation of double membrane vesicles called autophagosomes. Canonical signaling pathways, such as activation of the nutrient deprivation sensor AMP-activated protein kinase (AMPK) or inhibition of the nutrient-full sensor mechanistic target of rapamycin (mTOR) activate autophagy-related protein 1 (ATG1) kinase by phosphorylation. This in turns phosphorylates the Beclin 1 (BECN1) protein, allowing formation of a PtdIns3K complex that phosphorylates the phosphatidylinositol of intracellular membranes. Formation of the autophagosomes implicates the elongation of the membranes and their decoration with LC3 molecules, a process that is assisted by the ATG5 protein. The material targeted for degradation (i.e. proteins and organelles) arrives to the elongating membrane of the autophagosome through "receptors", like SQSTM1/p62, which binds polyubiquitinated proteins. Once the autophagosome is formed, it fuses with a lysosome that contains hydrolytic enzymes, leading to the degradation of the material enclosed within the autophagosome. The NFκB pathway is involved in transcriptional up-regulation of autophagy proteins such as BECN1, LC3 and ATG5, thereby promoting autophagy.

proteins that elongate the autophagosome (68). The mechanism of autophagy is depicted in **Figure 3**.

Autophagy may also be controlled by inflammatory pathways like those converging in the transcription factor NFκB (69–71). NFκB is a master regulator of the inflammatory response; the activation of this pathway depends on the degradation of its specific inhibitor, the inhibitor of NFκB (IκB) proteins following their phosphorylation by the IκB kinase IKK complex, allowing NFκB translocation to the nucleus that promotes the expression of genes involved in inflammation, cell proliferation and survival, epithelial-to-mesenchymal transition and invasion, angiogenesis and metastasis (72). Activation of NFκB induces expression of genes involved in autophagy such as BECN1, ATG5 and LC3, leading to increased autophagy (73, 74). However, NFκB may also reduce autophagy by promoting expression of mTOR pathway components (75). Most of the increased NFκB activity observed in solid malignant tumors is due to increased production of IKK-activating cytokines, including tumor necrosis factor, TNF, and IL1β (76). The role of NFκB in autophagy is also shown in **Figure 3**.

Modulation of autophagy is relevant during the pathophysiological progression of cancer. It has been reported that autophagy has a dual role in cancer. Autophagy inhibition in normal tissues leads to tumor formation, while in established tumors increased autophagy is a mechanism that overcomes a nutrient-deficient environment and promotes tumor growth (4). Spontaneous formation of lung carcinoma and hepatocellular carcinoma is observed in mice with a heterologous deletion of BECN1, suggesting that some components of the autophagic machinery may behave as tumor suppressors (77). However, breast carcinoma cells knocked down for BECN1 show decreased tumor growth *in vivo*, indicating that in developed carcinoma cells, BECN1 behaves as an oncogene (78). Similar results have been observed for other ATG proteins, including ATG5 and ATG7 (79, 80).

This dual role of autophagy in cancer is far more complex when considering that tumor cells and non-tumor cells within the tumor microenvironment exhibit different autophagic status, and that autophagy levels of each may vary in time, explaining tumor initiation and progression. This is the case for OSCC, as it will be discussed in the following section.

THE ROLE OF AUTOPHAGY IN THE OSCC MICROENVIRONMENT

Autophagic impairment is mostly associated with poorer prognosis in patients with OSCC (81). SQSTM1/p62 is highly accumulated in patients with advanced OSCC, suggesting that autophagosomes do not fuse with the lysosome in tumor cells (82). Accumulation of SQSTM1/p62, as well as LC3 and BECN1 in poorly differentiated OSCC, is correlated with immune infiltration of T cells and TAMs, revealing that autophagic inhibition during advanced stages of the OSCC is relevant to establish a tumor immune niche (83). Autophagy seems to inhibit OSCC cell migration, which may explain the reduced autophagic status during advanced OSCC. Indeed, downregulation of ATG7 in OSCC cells augments tumor cell migration through a mechanism

dependent on Toll-like receptor 4, TLR4, a protein highly expressed during poor prognosis OSCC (84). TLR4 may activate the NFκB pathway by inhibiting autophagy in OSCC cells (84). However, other studies suggest that autophagy activation is associated with the progression and poor prognosis in OSCC, because higher levels of BECN1, LC3, ATG5, and ATG16L are found in patients with advanced TNM stages (85–88). These observations, however, may be a result of either increased or decreased autophagy, and functional experiments would be required to clearly identify whether autophagy is activated or inhibited instead.

With low oxygen available and intracellular inhibition of autophagy, the preferred metabolic pathway in OSCC cells is glycolysis. High levels of the glycolytic enzyme phosphofructokinase, PFK, are commonly observed in poorly differentiated OSCCs (89). Novel compounds based on a 4H-1-benzopyran-4-ones structure show differential cytotoxicity on OSCC cells by blocking glycolysis, and on CAFs by suppressing the Krebs cycle (90), further indicating that the tumor microenvironment of OSCC depicts different metabolic requirements. Studies suggest that this metabolic difference occurs as a result of a cellular reprograming of normal fibroblasts into CAFs by OSCC cells (91). During this reprogramming, normal fibroblasts export their mitochondria into OSCC cells through tunneling nanotubes, which in turn produce lactate that fuels the fibroblasts and increases HIF1α-dependent transcription, supporting the transformation of normal fibroblasts into CAFs (91). Despite this mitochondrial transfer mechanism, OSCC relies on anaerobic metabolism to produce lactate. AMPK is inactive in the normal fibroblast. However, AMPK activity increases progressively when normal fibroblasts are transformed in CAFs, suggesting that autophagy may be an important factor in the formation of CAFs by OSCC cells (91). CAFs provoke a reduction in the activation of AMPK in OSCC cells, which explains not only the possibly reduced autophagy, but also the resistance to treatment against metformin, a chemical activator of AMPK that increases AMP levels within the cell and reduces tumor development in pancreatic, colorectal and hepatocellular cancer, among others (92, 93).

The low-to-high autophagy in CAFs is interesting and might explain the controversial role of autophagy in chemokine release from OSCC-related CAFs. Autophagy may be required for chemokine release. For instance, chemical inhibition of heat shock protein 90, HSP90, which is well-known to inhibit autophagy by decreasing NFκB-dependent transcription of autophagic genes like *beclin-1* in other cancer models (94), dramatically reduces the release of the CCL7 chemokine by CAFs, thereby decreasing the invasive rate of OSCC cells (95). As mentioned, the NFκB pathway is severely increased in CAFs as a result of IL1β stimulation (derived from OSCC cells), ultimately leading to the release of chemokines from CAFs (38). This suggests that HSP90-mediated autophagy in CAFs may be required for the release of chemokines that promote migration and invasion of OSCC cells. CCL2 and CXCL1 chemokines are released from skin keratinocytes after UV exposure by an ATG7-dependent mechanism (96). Inhibition of autophagy has been related to increased release of IL1β in macrophages (97), while CAF-related chemokines like CCL2 are known to reduce autophagy in breast cancer (98). There may be a vicious cycle between OSCC cells and CAFs; OSCC cells may release IL1β and promote autophagic-dependent release of chemokines by CAFs, which then inhibit autophagy in OSCC cells, raising the levels of IL1β even more. Further experiments would be required to confirm these possibilities.

Autophagy inhibition in OSCC cells can also be regulated by chemokines released from CAFs. For instance, expression of the FLJ22447 long non-coding RNA, lncRNA, is upregulated in CAFs, which prevents autophagic degradation of IL33 by inhibiting SQSTM1/p62 complex formation with the cargos in OSCC cells (26). Given that IL33 supports tumor growth (99), FLJ22447 is an important factor for OSCC cell proliferation (26), indicating that autophagy inhibition in CAFs is relevant for OSCC tumor growth.

Given that CAFs can both inhibit and activate autophagy in OSCC, a conciliated model is proposed, where immature CAFs, with low autophagy, may promote the release of some molecules such as IL33, while mature CAFs, with high autophagy, may promote the release of other cytokines like CCL2. Most importantly, given that OSCC cells direct CAF maturation, OSCC cells up-regulate their growth by modulating autophagy levels in CAFs. This interplay between CAFs and OSCC cells can provide therapeutic cancer resistance. *In vitro* exposure of OSCC cells to cellular stressors such as cadmium or tri-gas hypoxia leads to increased autophagy through mTOR inactivation (100, 101), suggesting that OSCC cells are still sensitive to autophagy under insults. Indeed, the lower basal autophagy in OSCC cells seems to provide a faster and sustained increase in autophagy after treatment with chemotherapeutic agents, such as cisplatin (102). Thus, an essential aspect to improve the chemotherapeutic toxicity over OSCC cells is to deplete the survival response of the tumor cells by concomitantly using an autophagy inhibitor compound like 3-methyladenine (102). The same results have been observed when OSCC cells are challenged with nutrient starvation conditions and co-treated with autophagy inhibitors (103). Given that CAFs reduce autophagy of OSCC cells, it is fair to conclude that CAFs promote the phenotype change of OSCC cells toward treatment-resistant cells. The phenotypic change of OSCC cells is associated with the expression of the membrane marker CD24, which correlates with both chemotherapy resistance and autophagy sensitivity (104). Curiously, while chemotherapy increases autophagy in OSCC cells, it decreases autophagy in CAFs, as reflected by higher accumulation of SQSTM1/p62 levels in α-SMA-containing cells (105).

To date, the role of autophagy in OSCC-related TAMs remains unclear. However, two recent studies have shown that OSCC cells can secrete high levels of the receptor activator of nuclear factor κB ligand, RANKL, a protein that transforms macrophage-derived pre-osteoclasts into osteoclasts, therefore promoting bone resorption and metastasis (106, 107). Interestingly, RANKL also binds to its receptor on OSCC cells, leading to a slight increase in autophagy that provides resistance against extrinsic apoptotic pathway inducers like the tumor necrosis factor-related apoptosis-inducing ligand, TRAIL (108). Caspase activation is observed when OSCC cells are stimulated with TRAIL in the absence of RANKL (108). This supports the view that reduced

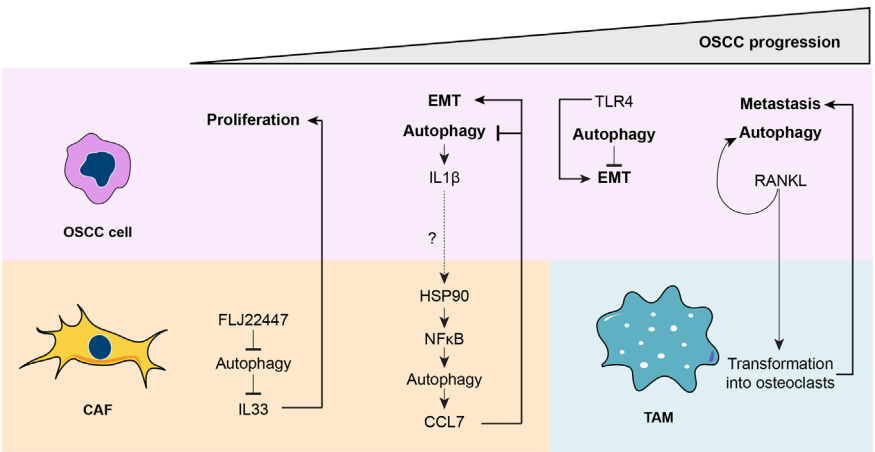


FIGURE 4 | A unified model for the role of autophagy during oral squamous cell carcinoma (OSCC) progression. During the early stages of OSCC, the long non-coding RNA FLJ22447 inhibits autophagy in cancer-associated fibroblasts (CAFs), impairing the autophagic degradation of IL33. Increased levels of IL33 are released from CAFs, inducing proliferation of the OSCC cells. Then, OSCC cells increase autophagy that releases interleukin-1β (IL1β), which then increases autophagy in CAFs through an nuclear factor κB ((NFκB)-dependent mechanism. The IL1β released from the OSCC cells may promote activation of heat shock protein 90 (HSP90) in the CAFs to increase NFκB activity, but this needs to be demonstrated (dashed line). However, it is known that NFκB-dependent autophagy in CAFs induces release of chemokine (C-C motif) ligand 7 (CCL7), which acts on OSCC cells, promoting epithelial-mesenchymal transition (EMT) and inhibiting autophagy. During later phases of OSCC, autophagy reduces Toll-like receptor 4 (TLR4)-dependent EMT. Therefore, inhibition of autophagy by CCL7 may promote TLR4-depedent EMT. Finally, during the advanced stages of OSCC, receptor activator of nuclear factor κB ligand (RANKL), which induces autophagy in OSCC cells, is released from OSCC cells transforming tumor-associated macrophages, TAMs, into osteoclasts, ultimately inducing metastasis.

basal autophagy in OSCC cells is required to respond promptly against a stressful condition and to cope with a death stimulus.

Together, autophagy is a key process to induce CAF maturation. The different levels of autophagy in CAFs provide a delicate tuning process that regulates the cytokines released in a time-specific manner. The maturation of CAFs is directed by OSCC cells; thereby OSCC cells control their malignancy by indirectly promoting the differential chemokine release from CAFs. Importantly, the lower the autophagic status of OSCC cells, the more malignant the tumor is and more resistant to chemotherapy. Increased sensitivity to cell death is achieved by co-treatment with chemotherapy and autophagy inhibitors, suggesting that malignant OSCC also respond better to autophagy-based treatments. Finally, OSCC cells release molecules that increase their autophagy status through an autocrine mechanism and modulate TAMs that promote metastasis. The role of autophagy during OSCC progression is depicted in **Figure 4**.

CONCLUDING REMARKS

The number of patients with OSCC keeps rising, because the risk factors involved in the development of OSCC are mainly environmental and considered to be "normal" by the modern society, such as tobacco smoking and alcohol consumption. Although OSCC is observed as morphological changes in oral epithelial cells, other cells in the oral tissue are also exposed to these risk factors. Efforts have been made during the last decade to elucidate the role of CAFs and immune cells in OSCC. These cells communicate with the OSCC cells and receive instructions from them by a complex cytokine interplay. Tobacco, alcohol and infection with HPV behave as direct tissue stressors modulating the homeostatic cellular process known as autophagy. Autophagic degradation levels are cell-specific and time-specific; OSCC cells show a counterintuitive reduction of autophagy over time, while CAFs show progressive increase in autophagy over time. This autophagic balance is important to induce the structural change from normal fibroblasts into CAFs that control the release of cytokines from CAFs and promote EMT in OSCC cells. Thus, autophagy is a critical player in the crosstalk between OSCC cells and tumor microenvironment cells such as CAFs and TAMs, fine-tuning the development of the OSCC.

AUTHOR CONTRIBUTIONS

Original draft writing by DP-O, MR, MH-C, CK, EM, SL, VT, and AC. Review and editing by DP-O, CAR-S, SL, VT, and AC. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Agencia Nacional de Investigación y Desarrollo (ANID, Chile): FONDECYT (3200313 to DP-O) (1200499 to EM) (1201684 to CAR-S) (1180495 to VT) (1171075 to AC); PIA-ANID (ACT172066 to EM and AC); FONDAP (15130011 to SL, VT, and AC); ANID PhD fellowship (21140848 to CK). Also supported by the U-Inicia Program of the Universidad de Chile (UI-024/19 to MR).

REFERENCES

1. Roma-Rodrigues C, Mendes R, Baptista PV, Fernandes AR. Targeting Tumor Microenvironment for Cancer Therapy. *Int J Mol Sci* (2019) 20 (4):840. doi: 10.3390/ijms20040840
2. Ribeiro Franco PI, Rodrigues AP, de Menezes LB, Pacheco Miguel M. Tumor microenvironment components: Allies of cancer progression. *Pathol Res Pract* (2020) 216(1):152729. doi: 10.1016/j.prp.2019.152729
3. Rivera C. Essentials of oral cancer. *Int J Clin Exp Pathol* (2015) 8(9):11884–94. doi: 10.5281/zenodo.192487
4. Singh SS, Vats S, Chia AY, Tan TZ, Deng S, Ong MS, et al. Dual role of autophagy in hallmarks of cancer. *Oncogene* (2018) 37(9):1142–58. doi: 10.1038/s41388-017-0046-6
5. Patil S, Rao RS, Raj AT. Dual Role of Autophagy in Oral Cancer. *J Int Oral Health* (2015) 7(6):i–ii.
6. Haddad RI, Shin DM. Recent advances in head and neck cancer. *N Engl J Med* (2008) 359(11):1143–54. doi: 10.1056/NEJMra0707975
7. Bishop JA, Sciubba JJ, Westra WH. Squamous Cell Carcinoma of the Oral Cavity and Oropharynx. *Surg Pathol Clin* (2011) 4(4):1127–51. doi: 10.1016/j.path.2011.07.002
8. Warnakulasuriya S. Living with oral cancer: epidemiology with particular reference to prevalence and life-style changes that influence survival. *Oral Oncol* (2010) 46(6):407–10. doi: 10.1016/j.oraloncology.2010.02.015
9. Zini A, Czerninski R, Sgan-Cohen HD. Oral cancer over four decades: epidemiology, trends, histology, and survival by anatomical sites. *J Oral Pathol Med* (2010) 39(4):299–305. doi: 10.1111/j.1600-0714.2009.00845.x
10. Montero PH, Patel SG. Cancer of the oral cavity. *Surg Oncol Clin N Am* (2015) 24(3):491–508. doi: 10.1016/j.soc.2015.03.006
11. Petersen PE. Oral cancer prevention and control–the approach of the World Health Organization. *Oral Oncol* (2009) 45(4-5):454–60. doi: 10.1016/j.oraloncology.2008.05.023
12. Spence T, Bruce J, Yip KW, Liu FF. HPV Associated Head and Neck Cancer. *Cancers (Basel)* (2016) 8(8):75. doi: 10.3390/cancers8080075
13. Oji C, Chukwuneke F. Poor oral Hygiene may be the Sole Cause of Oral Cancer. *J Maxillofac Oral Surg* (2012) 11(4):379–83. doi: 10.1007/s12663-012-0359-5
14. Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. *Nat Rev Cancer* (2011) 11(1):9–22. doi: 10.1038/nrc2982
15. Schaal C, Chellappan SP. Nicotine-mediated cell proliferation and tumor progression in smoking-related cancers. *Mol Cancer Res* (2014) 12(1):14–23. doi: 10.1158/1541-7786.MCR-13-0541
16. Cervigne NK, Machado J, Goswami RS, Sadikovic B, Bradley G, Perez-Ordonez B, et al. Recurrent genomic alterations in sequential progressive leukoplakia and oral cancer: drivers of oral tumorigenesis? *Hum Mol Genet* (2014) 23(10):2618–28. doi: 10.1093/hmg/ddt657
17. El-Naaj IA, Leiser Y, Shveis M, Sabo E, Peled M. Incidence of oral cancer occult metastasis and survival of T1-T2N0 oral cancer patients. *J Oral Maxillofac Surg* (2011) 69(10):2674–9. doi: 10.1016/j.joms.2011.02.012
18. Irani S. Distant metastasis from oral cancer: A review and molecular biologic aspects. *J Int Soc Prev Community Dent* (2016) 6(4):265–71. doi: 10.4103/2231-0762.186805
19. Chatzistefanou I, Lubek J, Markou K, Ord RA. The role of neck dissection and postoperative adjuvant radiotherapy in cN0 patients with PNI-positive squamous cell carcinoma of the oral cavity. *Oral Oncol* (2014) 50(8):753–8. doi: 10.1016/j.oraloncology.2014.05.005
20. Genden EM, Ferlito A, Silver CE, Takes RP, Suárez C, Owen RP, et al. Contemporary management of cancer of the oral cavity. *Eur Arch Otorhinolaryngol* (2010) 267(7):1001–17. doi: 10.1007/s00405-010-1206-2
21. Shah S, Kaur M. Biomarkers and chemopreventives in oral carcinogenesis and its prevention. *J Oral Maxillofac Pathol* (2014) 18(1):69–76. doi: 10.4103/0973-029X.131914
22. Dourado MR, Guerra ENS, Salo T, Lambert DW, Coletta RD. Prognostic value of the immunohistochemical detection of cancer-associated fibroblasts in oral cancer: A systematic review and meta-analysis. *J Oral Pathol Med* (2018) 47(5):443–53. doi: 10.1111/jop.12623
23. Dourado MR, de Oliveira CE, Sawazaki-Calone I, Sundquist E, Coletta RD, Salo T. Clinicopathologic significance of ROCK2 expression in oral squamous cell carcinomas. *J Oral Pathol Med* (2018) 47(2):121–7. doi: 10.1111/jop.12651
24. Min A, Zhu C, Wang J, Peng S, Shuai C, Gao S, et al. Focal adhesion kinase knockdown in carcinoma-associated fibroblasts inhibits oral squamous cell carcinoma metastasis via downregulating MCP-1/CCL2 expression. *J Biochem Mol Toxicol* (2015) 29(2):70–6. doi: 10.1002/jbt.21669
25. Wang Y, Jing Y, Ding L, Zhang X, Song Y, Chen S, et al. Epiregulin reprograms cancer-associated fibroblasts and facilitates oral squamous cell carcinoma invasion via JAK2-STAT3 pathway. *J Exp Clin Cancer Res* (2019) 38(1):274. doi: 10.1186/s13046-019-1277-x
26. Ding L, Ren J, Zhang D, Li Y, Huang X, Hu Q, et al. A novel stromal lncRNA signature reprograms fibroblasts to promote the growth of oral squamous cell carcinoma via LncRNA-CAF/interleukin-33. *Carcinogenesis* (2018) 39 (3):397–406. doi: 10.1093/carcin/bgy006
27. Kartha VK, Stawski L, Han R, Haines P, Gallagher G, Noonan V, et al. PDGFRβ Is a Novel Marker of Stromal Activation in Oral Squamous Cell Carcinomas. *PLoS One* (2016) 11(4):e0154645. doi: 10.1371/journal.pone.0154645
28. Dourado MR, Korvala J, Åström P, De Oliveira CE, Cervigne NK, Mofatto LS, et al. Extracellular vesicles derived from cancer-associated fibroblasts induce the migration and invasion of oral squamous cell carcinoma. *J Extracell Vesicles* (2019) 8(1):1578525. doi: 10.1080/20013078.2019.1578525
29. Sun LP, Xu K, Cui J, Yuan DY, Zou B, Li J, et al. Cancer−associated fibroblast −derived exosomal miR−382−5p promotes the migration and invasion of oral squamous cell carcinoma. *Oncol Rep* (2019) 42(4):1319–28. doi: 10.3892/or.2019.7255
30. Zhang X, Zhao H, Zhang Y, Yang X, Zhang J, Yi M, et al. The MicroRNA-382-5p/MXD1 Axis Relates to Breast Cancer Progression and Promotes Cell Malignant Phenotypes. *J Surg Res* (2020) 246:442–9. doi: 10.1016/j.jss.2019.09.018
31. Du J, Bai F, Zhao P, Li X, Li X, Gao L, et al. Hepatitis B core protein promotes liver cancer metastasis through miR-382-5p/DLC-1 axis. *Biochim Biophys Acta Mol Cell Res* (2018) 1865(1):1–11. doi: 10.1016/j.bbamcr.2017.09.020
32. Li YY, Tao YW, Gao S, Li P, Zheng JM, Zhang SE, et al. Cancer-associated fibroblasts contribute to oral cancer cells proliferation and metastasis via exosome-mediated paracrine miR-34a-5p. *EBioMedicine* (2018) 36:209–20. doi: 10.1016/j.ebiom.2018.09.006
33. Jung DW, Che ZM, Kim J, Kim K, Kim KY, Williams D, et al. Tumor-stromal crosstalk in invasion of oral squamous cell carcinoma: a pivotal role of CCL7. *Int J Cancer* (2010) 127(2):332–44. doi: 10.1002/ijc.25060
34. Fujita S, Ikeda T. The CCL2-CCR2 Axis in Lymph Node Metastasis From Oral Squamous Cell Carcinoma: An Immunohistochemical Study. *J Oral Maxillofac Surg* (2017) 75(4):742–9. doi: 10.1016/j.joms.2016.09.052
35. Li X, Xu Q, Wu Y, Li J, Tang D, Han L, et al. A CCL2/ROS autoregulation loop is critical for cancer-associated fibroblasts-enhanced tumor growth of oral squamous cell carcinoma. *Carcinogenesis* (2014) 35(6):1362–70. doi: 10.1093/carcin/bgu046
36. Kathiriya JJ, Nakra N, Nixon J, Patel PS, Vaghasiya V, Alhassani A, et al. Galectin-1 inhibition attenuates profibrotic signaling in hypoxia-induced pulmonary fibrosis. *Cell Death Discov* (2017) 3:17010. doi: 10.1038/cddiscovery.2017.10
37. Wu MH, Hong HC, Hong TM, Chiang WF, Jin YT, Chen YL. Targeting galectin-1 in carcinoma-associated fibroblasts inhibits oral squamous cell carcinoma metastasis by downregulating MCP-1/CCL2 expression. *Clin Cancer Res* (2011) 17(6):1306–16. doi: 10.1158/1078-0432.CCR-10-1824
38. Wei LY, Lee JJ, Yeh CY, Yang CJ, Kok SH, Ko JY, et al. Reciprocal activation of cancer-associated fibroblasts and oral squamous carcinoma cells through CXCL1. *Oral Oncol* (2019) 88:115–23. doi: 10.1016/j.oraloncology.2018.11.002
39. Kim EK, Moon S, Kim DK, Zhang X, Kim J. CXCL1 induces senescence of cancer-associated fibroblasts via autocrine loops in oral squamous cell carcinoma. *PLoS One* (2018) 13(1):e0188847. doi: 10.1371/journal.pone.0188847
40. Takahashi H, Sakakura K, Kudo T, Toyoda M, Kaira K, Oyama T, et al. Cancer-associated fibroblasts promote an immunosuppressive microenvironment through the induction and accumulation of protumoral macrophages. *Oncotarget* (2017) 8(5):8633–47. doi: 10.18632/oncotarget.14374

41. Alves AM, Diel LF, Lamers ML. Macrophages and prognosis of oral squamous cell carcinoma: A systematic review. *J Oral Pathol Med* (2018) 47(5):460–7. doi: 10.1111/jop.12643
42. Fabriek BO, Dijkstra CD, van den Berg TK. The macrophage scavenger receptor CD163. *Immunobiology* (2005) 210(2-4):153–60. doi: 10.1016/j.imbio.2005.05.010
43. Gomes FG, Nedel F, Alves AM, Nör JE, Tarquinio SB. Tumor angiogenesis and lymphangiogenesis: tumor/endothelial crosstalk and cellular/microenvironmental signaling mechanisms. *Life Sci* (2013) 92(2):101–7. doi: 10.1016/j.lfs.2012.10.008
44. Fujii N, Shomori K, Shiomi T, Nakabayashi M, Takeda C, Ryoke K, et al. Cancer-associated fibroblasts and CD163-positive macrophages in oral squamous cell carcinoma: their clinicopathological and prognostic significance. *J Oral Pathol Med* (2012) 41(6):444–51. doi: 10.1111/j.1600-0714.2012.01127.x
45. Haque A, Moriyama M, Kubota K, Ishiguro N, Sakamoto M, Chinju A, et al. CD206(+) tumor-associated macrophages promote proliferation and invasion in oral squamous cell carcinoma via EGF production. *Sci Rep* (2019) 9(1):14611. doi: 10.1038/s41598-019-51149-1
46. Meng W, Xia Q, Wu L, Chen S, He X, Zhang L, et al. Downregulation of TGF-beta receptor types II and III in oral squamous cell carcinoma and oral carcinoma-associated fibroblasts. *BMC Cancer* (2011) 11:88. doi: 10.1186/1471-2407-11-88
47. Pickup M, Novitskiy S, Moses HL. The roles of TGFβ in the tumour microenvironment. *Nat Rev Cancer* (2013) 13(11):788–99. doi: 10.1038/nrc3603
48. Zhao X, Ding L, Lu Z, Huang X, Jing Y, Yang Y, et al. Diminished CD68(+) Cancer-Associated Fibroblast Subset Induces Regulatory T-Cell (Treg) Infiltration and Predicts Poor Prognosis of Oral Squamous Cell Carcinoma Patients. *Am J Pathol* (2020) 190(4):886–99. doi: 10.1016/j.ajpath.2019.12.007
49. Gasparoto TH, de Souza Malaspina TS, Benevides L, de Melo EJ Jr., Costa MR, Damante JH, et al. Patients with oral squamous cell carcinoma are characterized by increased frequency of suppressive regulatory T cells in the blood and tumor microenvironment. *Cancer Immunol Immunother* (2010) 59(6):819–28. doi: 10.1007/s00262-009-0803-7
50. Tang CH, Chuang JY, Fong YC, Maa MC, Way TD, Hung CH. Bone-derived SDF-1 stimulates IL-6 release via CXCR4, ERK and NF-kappaB pathways and promotes osteoclastogenesis in human oral cancer cells. *Carcinogenesis* (2008) 29(8):1483–92. doi: 10.1093/carcin/bgn045
51. Punyani SR, Sathawane RS. Salivary level of interleukin-8 in oral precancer and oral squamous cell carcinoma. *Clin Oral Invest* (2013) 17(2):517–24. doi: 10.1007/s00784-012-0723-3
52. Tsunoda K, Tsujino I, Koshi R, Sugano N, Sato S, Asano M. Nicotine-Mediated Ca(2+)-Influx Induces IL-8 Secretion in Oral Squamous Cell Carcinoma Cell. *J Cell Biochem* (2016) 117(4):1009–15. doi: 10.1002/jcb.25387
53. Kaur J, Debnath J. Autophagy at the crossroads of catabolism and anabolism. *Nat Rev Mol Cell Biol* (2015) 16(8):461–72. doi: 10.1038/nrm4024
54. Murrow L, Debnath J. Autophagy as a stress-response and quality-control mechanism: implications for cell injury and human disease. *Annu Rev Pathol* (2013) 8:105–37. doi: 10.1146/annurev-pathol-020712-163918
55. Kim MS, Yun JW, Park JH, Park BW, Kang YH, Hah YS, et al. Autophagy Has a Beneficial Role in Relieving Cigarette Smoke-Induced Apoptotic Death in Human Gingival Fibroblasts. *Int J Med Sci* (2016) 13(5):357–64. doi: 10.7150/ijms.14592
56. Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. *Cell Res* (2014) 24(1):24–41. doi: 10.1038/cr.2013.168
57. Yang Y, Klionsky DJ. Autophagy and disease: unanswered questions. *Cell Death Differ* (2020) 27(3):858–71. doi: 10.1038/s41418-019-0480-9
58. Tanida I, Ueno T, Kominami E. LC3 and Autophagy. *Methods Mol Biol* (2008) 445:77–88. doi: 10.1007/978-1-59745-157-4_4
59. Tanida I, Ueno T, Kominami E. Human light chain 3/MAP1LC3B is cleaved at its carboxyl-terminal Met121 to expose Gly120 for lipidation and targeting to autophagosomal membranes. *J Biol Chem* (2004) 279 (46):47704–10. doi: 10.1074/jbc.M407016200
60. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* (2016) 12(1):1–222. doi: 10.1080/15548627.2015.1100356
61. Yoshii SR, Mizushima N. Monitoring and Measuring Autophagy. *Int J Mol Sci* (2017) 18(9):1865. doi: 10.3390/ijms18091865
62. Johansen T, Lamark T. Selective autophagy mediated by autophagic adapter proteins. *Autophagy* (2011) 7(3):279–96. doi: 10.4161/auto.7.3.14487
63. Bjørkøy G, Lamark T, Pankiv S, Øvervatn A, Brech A, Johansen T. Monitoring autophagic degradation of p62/SQSTM1. *Methods Enzymol* (2009) 452:181–97. doi: 10.1016/S0076-6879(08)03612-4
64. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol* (2011) 13(2):132–41. doi: 10.1038/ncb2152
65. Li L, Chen Y, Gibson SB. Starvation-induced autophagy is regulated by mitochondrial reactive oxygen species leading to AMPK activation. *Cell Signal* (2013) 25(1):50–65. doi: 10.1016/j.cellsig.2012.09.020
66. Meijer AJ, Lorin S, Blommaart EF, Codogno P. Regulation of autophagy by amino acids and MTOR-dependent signal transduction. *Amino Acids* (2015) 47(10):2037–63. doi: 10.1007/s00726-014-1765-4
67. Axe EL, Walker SA, Manifava M, Chandra P, Roderick HL, Habermann A, et al. Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. *J Cell Biol* (2008) 182(4):685–701. doi: 10.1083/jcb.200803137
68. Russell RC, Tian Y, Yuan H, Park HW, Chang YY, Kim J, et al. ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. *Nat Cell Biol* (2013) 15(7):741–50. doi: 10.1038/ncb2757
69. Niso-Santano M, Criollo A, Malik SA, Michaud M, Morselli E, Mariño G, et al. Direct molecular interactions between Beclin 1 and the canonical NFκB activation pathway. *Autophagy* (2012) 8(2):268–70. doi: 10.4161/auto.8.2.18845
70. Criollo A, Senovilla L, Authier H, Maiuri MC, Morselli E, Vitale I, et al. IKK connects autophagy to major stress pathways. *Autophagy* (2010) 6(1):189–91. doi: 10.4161/auto.6.1.10818
71. Criollo A, Senovilla L, Authier H, Maiuri MC, Morselli E, Vitale I, et al. The IKK complex contributes to the induction of autophagy. *EMBO J* (2010) 29 (3):619–31. doi: 10.1038/emboj.2009.364
72. Liu T, Zhang L, Joo D, Sun SC. NF-κB signaling in inflammation. *Signal Transduct Target Ther* (2017) 2:17023–. doi: 10.1038/sigtrans.2017.23
73. Copetti T, Bertoli C, Dalla E, Demarchi F, Schneider C. p65/RelA modulates BECN1 transcription and autophagy. *Mol Cell Biol* (2009) 29(10):2594–608. doi: 10.1128/MCB.01396-08
74. Nivon M, Richet E, Codogno P, Arrigo AP, Kretz-Remy C. Autophagy activation by NFkappaB is essential for cell survival after heat shock. *Autophagy* (2009) 5(6):766–83. doi: 10.4161/auto.8788
75. Lee DF, Kuo HP, Chen CT, Hsu JM, Chou CK, Wei Y, et al. IKK beta suppression of TSC1 links inflammation and tumor angiogenesis via the mTOR pathway. *Cell* (2007) 130(3):440–55. doi: 10.1016/j.cell.2007.05.058
76. Xia Y, Shen S, Verma IM. NF-κB, an active player in human cancers. *Cancer Immunol Res* (2014) 2(9):823–30. doi: 10.1158/2326-6066.CIR-14-0112
77. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest* (2003) 112(12):1809–20. doi: 10.1172/JCI20039
78. Gong C, Bauvy C, Tonelli G, Yue W, Deloménie C, Nicolas V, et al. Beclin 1 and autophagy are required for the tumorigenicity of breast cancer stem-like/progenitor cells. *Oncogene* (2013) 32(18):2261–72. doi: 10.1038/onc.2012.252
79. Zhu J, Li Y, Tian Z, Hua X, Gu J, Li J, et al. ATG7 Overexpression Is Crucial for Tumorigenic Growth of Bladder Cancer In Vitro and In Vivo by Targeting the ETS2/miRNA196b/FOXO1/p27 Axis. *Mol Ther Nucleic Acids* (2017) 7:299–313. doi: 10.1016/j.omtn.2017.04.012
80. Cho DH, Jo YK, Kim SC, Park IJ, Kim JC. Down-regulated expression of ATG5 in colorectal cancer. *Anticancer Res* (2012) 32(9):4091–6.
81. Inui T, Chano T, Takikita-Suzuki M, Nishikawa M, Yamamoto G, Okabe H. Association of p62/SQSTM1 excess and oral carcinogenesis. *PLoS One* (2013) 8(9):e74398. doi: 10.1371/journal.pone.0074398
82. Liu JL, Chen FF, Lung J, Lo CH, Lee FH, Lu YC, et al. Prognostic significance of p62/SQSTM1 subcellular localization and LC3B in oral squamous cell carcinoma. *Br J Cancer* (2014) 111(5):944–54. doi: 10.1038/bjc.2014.355
83. Sakakura K, Takahashi H, Kaira K, Toyoda M, Oyama T, Chikamatsu K. Immunological significance of the accumulation of autophagy components in oral squamous cell carcinoma. *Cancer Sci* (2015) 106(1):1–8. doi: 10.1111/cas.12559

84. Kong Q, Liang Y, He Q, You Y, Wu L, Liang L, et al. Autophagy inhibits TLR4-mediated invasiveness of oral cancer cells via the NF-κB pathway. *Oral Dis* (2020) 26(6):1165–74. doi: 10.1111/odi.13355
85. Wang Y, Wang X, Zhang Y, Yu L, Zhu B, Wu S, et al. Vasculogenic mimicry and expression of ALDH1, Beclin1, and p16 correlate with metastasis and prognosis in oral squamous cell carcinoma. *Int J Clin Exp Pathol* (2018) 11 (3):1599–609.
86. Tang JY, Fang YY, Hsi E, Huang YC, Hsu NC, Yang WC, et al. Immunopositivity of Beclin-1 and ATG5 as indicators of survival and disease recurrence in oral squamous cell carcinoma. *Anticancer Res* (2013) 33(12):5611–6.
87. Tang JY, Hsi E, Huang YC, Hsu NC, Chu PY, Chai CY. High LC3 expression correlates with poor survival in patients with oral squamous cell carcinoma. *Hum Pathol* (2013) 44(11):2558–62. doi: 10.1016/j.humpath.2013.06.017
88. Tang JY, Hsi E, Huang YC, Hsu NC, Yang WC, Chang HW, et al. Overexpression of autophagy-related 16-like 1 in patients with oral squamous cell carcinoma. *Pathol Oncol Res* (2015) 21(2):301–5. doi: 10.1007/s12253-014-9821-7
89. Chen G, Liu H, Zhang Y, Liang J, Zhu Y, Zhang M, et al. Silencing PFKP inhibits starvation-induced autophagy, glycolysis, and epithelial mesenchymal transition in oral squamous cell carcinoma. *Exp Cell Res* (2018) 370(1):46–57. doi: 10.1016/j.yexcr.2018.06.007
90. Sakagami H, Shimada C, Kanda Y, Amano O, Sugimoto M, Ota S, et al. Effects of 3-styrylchromones on metabolic profiles and cell death in oral squamous cell carcinoma cells. *Toxicol Rep* (2015) 2:1281–90. doi: 10.1016/j.toxrep.2015.09.009
91. Zhang Z, Gao Z, Rajthala S, Sapkota D, Dongre H, Parajuli H, et al. Metabolic reprogramming of normal oral fibroblasts correlated with increased glycolytic metabolism of oral squamous cell carcinoma and precedes their activation into carcinoma associated fibroblasts. *Cell Mol Life Sci* (2020) 77(6):1115–33. doi: 10.1007/s00018-019-03209-y
92. Zhang Z, Liang X, Fan Y, Gao Z, Bindoff LA, Costea DE, et al. Fibroblasts rescue oral squamous cancer cell from metformin-induced apoptosis via alleviating metabolic disbalance and inhibiting AMPK pathway. *Cell Cycle* (2019) 18(9):949–62. doi: 10.1080/15384101.2019.1598727
93. Zi F, Zi H, Li Y, He J, Shi Q, Cai Z. Metformin and cancer: An existing drug for cancer prevention and therapy. *Oncol Lett* (2018) 15(1):683–90. doi: 10.3892/ol.2017.7412
94. Jiang Q, Wang Y, Li T, Shi K, Li Z, Ma Y, et al. Heat shock protein 90-mediated inactivation of nuclear factor-κB switches autophagy to apoptosis through becn1 transcriptional inhibition in selenite-induced NB4 cells. *Mol Biol Cell* (2011) 22(8):1167–80. doi: 10.1091/mbc.e10-10-0860
95. Jung DW, Kim J, Che ZM, Oh ES, Kim G, Eom SH, et al. A triazine compound S06 inhibits proinvasive crosstalk between carcinoma cells and stromal fibroblasts via binding to heat shock protein 90. *Chem Biol* (2011) 18 (12):1581–90. doi: 10.1016/j.chembiol.2011.10.001
96. Qiang L, Sample A, Shea CR, Soltani K, Macleod KF, He YY. Autophagy gene ATG7 regulates ultraviolet radiation-induced inflammation and skin tumorigenesis. *Autophagy* (2017) 13(12):2086–103. doi: 10.1080/15548627.2017.1380757
97. Dupont N, Jiang S, Pilli M, Ornatowski W, Bhattacharya D, Deretic V. Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1β. *EMBO J* (2011) 30(23):4701–11. doi: 10.1038/emboj.2011.398
98. Fang WB, Yao M, Jokar I, Alhakamy N, Berkland C, Chen J, et al. The CCL2 chemokine is a negative regulator of autophagy and necrosis in luminal B breast cancer cells. *Breast Cancer Res Treat* (2015) 150(2):309–20. doi: 10.1007/s10549-015-3324-4
99. Kudo-Saito C, Miyamoto T, Imazeki H, Shoji H, Aoki K, Boku N. IL33 Is a Key Driver of Treatment Resistance of Cancer. *Cancer Res* (2020) 80 (10):1981–90. doi: 10.1158/0008-5472.CAN-19-2235
100. Fan T, Chen Y, He Z, Wang Q, Yang X, Ren Z, et al. Inhibition of ROS/NUPR1-dependent autophagy antagonises repeated cadmium exposure -induced oral squamous cell carcinoma cell migration and invasion. *Toxicol Lett* (2019) 314:142–52. doi: 10.1016/j.toxlet.2019.07.017
101. Gao L, Dou ZC, Ren WH, Li SM, Liang X, Zhi KQ. CircCDR1as upregulates autophagy under hypoxia to promote tumor cell survival via AKT/ERK (½)/mTOR signaling pathways in oral squamous cell carcinomas. *Cell Death Dis* (2019) 10(10):745. doi: 10.1038/s41419-019-1971-9
102. Li S, Wu Y, Ding Y, Yu M, Ai Z. CerS6 regulates cisplatin resistance in oral squamous cell carcinoma by altering mitochondrial fission and autophagy. *J Cell Physiol* (2018) 233(12):9416–25. doi: 10.1002/jcp.26815
103. Jiang LC, Xin ZY, Deborah B, Zhang JS, Yuan DY, Xu K, et al. Inhibition of autophagy augments apoptosis in human oral squamous cell carcinoma under nutrient depletion. *J Oral Pathol Med* (2015) 44(5):361–6. doi: 10.1111/jop.12250
104. Biddle A, Gammon L, Liang X, Costea DE, Mackenzie IC. Phenotypic Plasticity Determines Cancer Stem Cell Therapeutic Resistance in Oral Squamous Cell Carcinoma. *EBioMedicine* (2016) 4:138–45. doi: 10.1016/j.ebiom.2016.01.007
105. Liang L, Luo H, He Q, You Y, Fan Y, Liang J. Investigation of cancer-associated fibroblasts and p62 expression in oral cancer before and after chemotherapy. *J Craniomaxillofac Surg* (2018) 46(4):605–10. doi: 10.1016/j.jcms.2017.12.016
106. Sambandam Y, Ethiraj P, Hathaway-Schrader JD, Novince CM, Panneerselvam E, Sundaram K, et al. Autoregulation of RANK ligand in oral squamous cell carcinoma tumor cells. *J Cell Physiol* (2018) 233(8):6125–34. doi: 10.1002/jcp.26456
107. Zhang X, Junior CR, Liu M, Li F, D'Silva NJ, Kirkwood KL. Oral squamous carcinoma cells secrete RANKL directly supporting osteolytic bone loss. *Oral Oncol* (2013) 49(2):119–28. doi: 10.1016/j.oraloncology.2012.08.004
108. Ethiraj P, Sambandam Y, Hathaway-Schrader JD, Haque A, Novince CM, Reddy SV. RANKL triggers resistance to TRAIL-induced cell death in oral squamous cell carcinoma. *J Cell Physiol* (2020) 235(2):1663–73. doi: 10.1002/jcp.29086

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Peña-Oyarzún, Reyes, Hernández-Cáceres, Kretschmar, Morselli, Ramirez-Sarmiento, Lavandero, Torres and Criollo. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 15 December 2020
doi: 10.3389/fcell.2020.606817

A Novel Autophagy-Related lncRNAs Signature for Prognostic Prediction and Clinical Value in Patients With Pancreatic Cancer

Zhengdong Deng[1†], Xiangyu Li[1†], Yuanxin Shi[1], Yun Lu[1], Wei Yao[2] and Jianming Wang[1,3*]*

[1] Department of Biliary and Pancreatic Surgery/Cancer Research Center Affiliated Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [2] Department of Oncology Affiliated Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [3] Affiliated Tianyou Hospital, Wuhan University of Science and Technology, Wuhan, China

OPEN ACCESS

Edited by:
Eugenia Morselli,
Pontificia Universidad Católica de Chile, Chile

Reviewed by:
Daniele Vergara,
University of Salento, Italy
Saber Imani,
Affiliated Hospital of Southwest Medical University, China

***Correspondence:**
Wei Yao
yw13557@163.com
Jianming Wang
wjm18jgm@aliyun.com

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: 15 September 2020
Accepted: 16 November 2020
Published: 15 December 2020

Citation:
Deng Z, Li X, Shi Y, Lu Y, Yao W and Wang J (2020) A Novel Autophagy-Related lncRNAs Signature for Prognostic Prediction and Clinical Value in Patients With Pancreatic Cancer. Front. Cell Dev. Biol. 8:606817. doi: 10.3389/fcell.2020.606817

Autophagy is an important bioprocess throughout the occurrence and development of cancer. However, the role of autophagy-related lncRNAs in pancreatic cancer (PC) remains obscure. In the study, we identified the autophagy-related lncRNAs (ARlncRNAs) and divided the PC patients from The Cancer Genome Atlas into training and validation set. Firstly, we constructed a signature in the training set by the least absolute shrinkage and selection operator penalized cox regression analysis and the multivariate cox regression analysis. Then, we validated the independent prognostic role of the risk signature in both training and validation set with survival analysis, receiver operating characteristic analysis, and Cox regression. The nomogram was established to demonstrate the predictive power of the signature. Moreover, high risk scores were significantly correlated to worse outcomes and severe clinical characteristics. The Pearson's analysis between risk scores with immune cells infiltration, tumor mutation burden, and the expression level of chemotherapy target molecules indicated that the signature could predict efficacy of immunotherapy and targeted therapy. Next, we constructed an lncRNA–miRNA–mRNA regulatory network and identified several potential small molecule drugs in the Connectivity Map (CMap). What's more, quantitative real-time PCR (qRT-PCR) analysis showed that serum LINC01559 could serve as a diagnostic biomarker. *In vitro* analysis showed inhibition of LINC01559 suppressed PC cell proliferation, migration, and invasion. Additionally, silencing LINC01559 suppressed gemcitabine-induced autophagy and promoted the sensitivity of PC cells to gemcitabine. In conclusion, we identified a novel ARlncRNAs signature with valuable clinical utility for reliable prognostic prediction and personalized treatment of PC patients. And inhibition of LINC01559 might be a novel strategy to overcome chemoresistance.

Keywords: pancreatic cancer, autophagy-related lncRNA signature, prognostic prediction, LINC01559, chemoresistance

Abbreviations: PC, pancreatic carcinoma; lncRNA, long non-coding RNA; ARlncRNA, autophagy related lncRNA; TCGA, The Cancer Genome Atlas; DEGs, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; ROC, receiver operating characteristic.

INTRODUCTION

Pancreatic cancer (PC) is one of the most lethal malignancies with a rising incidence and an extremely poor prognosis. There will be approximately 57,600 new PC cases and 47,050 deaths occurred in the United States in 2020 (Siegel et al., 2020). Although therapeutic treatments for PC have been improved, including surgery, chemotherapy, radiotherapy, and immunotherapy, 5 years survival rates remain unsatisfactory (Vincent et al., 2011; Bliss et al., 2014). Therefore, there is an urgent need to identify reliable biomarkers for the prognostic prediction and develop effective therapeutic strategies for PC patients.

Long non-coding RNA (lncRNA) is a gene transcription composed of more than 200 nucleotides, which has been reported to be aberrantly expressed and abnormally regulated in multiple cancers (Li et al., 2016; Castro-Oropeza et al., 2018). Accumulated evidence have shown that lncRNAs are involved in a variety of cancer biological processes, such as epigenetic regulation, DNA damage, immune escape, metabolic disorders, chemical resistance, as well as epithelial-mesenchymal transition (EMT), and cell stemness (Jiang et al., 2019). The underlying mechanism may be related to the mutual correction of lncRNA and other cellular molecules, including DNA, miRNA, and mRNA (Tang X. J. et al., 2019; Grixti and Ayers, 2020). At present, several lncRNAs have been identified as tumor biomarkers, such as HOTAIR, MALAT1, and H19. Iyer et al. (2015) curated a total of 7,256 RNA-seq libraries and identified 7,942 cancer-associated lncRNAs that could potentially be biomarkers for specific cancer types. Thus, better understanding of the role of lncRNAs in cancer is helpful to identify novel diagnostic biomarkers and develop potential therapeutic targets.

Autophagy, also known as type II cell death, is a process in which cells use lysosomes to degrade their damaged organelles and macromolecules under the regulation of autophagy related signaling pathways. Autophagy is involved in pathophysiological processes of multiple diseases, including neurodegenerative diseases, metabolic diseases, infectious diseases, and cancers (Yang et al., 2017). Flaks et al. (1981) first proposed the presence of autophagy during pancreatic carcinogenesis. Indeed, the role of autophagy in cancer is still controversial. Emerging evidence suggests that suppressed autophagy contributes to initiation of carcinogenesis, while activated autophagy is required for malignancy maintenance and development (Chen K. D. et al., 2018; Folkerts et al., 2019). Moreover, autophagy is reported to play a vital role in cancer cells survival, metastasis, and drug resistance (Yun and Lee, 2018). Several cellular molecules and signaling pathways are involved in autophagy regulation, including lncRNAs. Hu et al. reported that lncRNA MALAT1 regulated autophagy-related chemoresistance in gastric cancer (YiRen et al., 2017). However, the role of autophagy-related lncRNAs in PC has been not fully elaborated yet. Therefore, this study aimed to identify the autophagy-related lncRNAs and explore their clinical relevance in PC.

In the present study, we identified the autophagy-related lncRNAs of PC and established a risk model that could provide valuable clinical utility for prognostic prediction and potential drugs selection of PC patients.

MATERIALS AND METHODS

Data Acquisition and Processing

The pancreatic adenocarcinoma RNA-seq data and corresponding clinical information were downloaded from the TCGA dataset[1]. The cohort contains 178 tumor tissues and four normal pancreatic tissues. And, 177 PC patients with complete clinical information were extracted for further analysis. Perl language was performed to convert gene names from Ensemble IDs to a profile of gene symbols with the Ensemble database[2].

Identification of Autophagy-Related lncRNAs

Autophagy-related genes (ARGs) were obtained from the Human Autophagy Database[3]. We extracted the lncRNA list from mRNA expression data of the GNECODE project[4]. Then, the Pearson correlation was applied to analyze the correlation between the lncRNAs and ARGs. The lncRNAs with correlation coefficient $|R^2| > 0.5$ and $p < 0.01$ were considered as autophagy-related lncRNAs (ARlncRNAs).

Construction and Validation of an ARlncRNAs Prognostic Signature

To increase the reliability of our study, we randomly divided the entire dataset into a training set (accounting for 60%) and a validation set (accounting for 40%) by the "caret" R package (version 6.0-84)[5] (Deist et al., 2018). And the whole dataset was considered as an entire set ($n = 177$). At first, we adopted the univariate cox regression analysis to identify the significant ARlncRNAs in the training set with a $p < 0.01$ by the "survival" R package. Then, the least absolute shrinkage and selection operator (LASSO) penalized cox regression analysis was performed to further reduce the dimension and the multivariate cox regression analysis was utilized to calculate the risk coefficients of the prognostic signature. The risk score formula is shown as follows: Risk score = ΣCoef ARlncRNAs $\times$ Exp ARlncRNAs. The Coef ARlncRNAs represents the coefficient of each ARlncRNAs and Exp ARlncRNAs is the expression of each ARlncRNAs. Based on the median risk score of the signature, the patients were divided into low-risk and high-risk groups. The survival analysis for the different groups was performed using the Kaplan-Meier (K-M) survival curve analysis and log-rank test analysis with the "survminer" R package. Moreover, we constructed the receiver operating characteristic (ROC) curve by using the "survivalROC" R package to evaluate the specificity and sensitivity of the prognostic signature.

The Nomogram Establishing

In order to simplify the predictive model, we created a nomogram based on independent clinical prognostic factors with the "rms"

[1]https://portal.gdc.cancer.gov

[2]http://asia.ensembl.org/index.html

[3]http://autophagy.lu/clustering/index.html

[4]http://www.gencodegenes.org

[5]http://caret.r-forge.r-project.org/

R package (Iasonos et al., 2008). We plotted the calibration curve of the nomogram to value the predictive power of the prognostic signature.

Bioinformatics Analysis of the Signature

Grouped samples and expression patterns were analyzed using the principal component analysis (PCA). Gene set enrichment analysis (GSEA) was performed to evaluate different functional phenotypes between low- and high-risk groups (Subramanian et al., 2005). Moreover, we analyzed the correlation between different risk groups and clinical characteristics with the chi-square test and the results were presented in a heat map.

To better investigate the relationship between the signature and immune cell infiltration, we calculated the infiltration expression of 22 immune cells in PC by using the "CIBERSORT" R package. Then, the immune-related Pearson correlation coefficients were tested for relevance in the R program.

Moreover, to explore the clinical utility of the signature to predict therapeutic effect, we used the Pearson's analysis to calculate the correlation between risk scores with molecules of targeted therapy. The therapy targets are as follows: programmed cell death 1 (PD-1, also known as PCDC1), programmed cell death ligand 1 (PD-L1, also known as CD274), epidermal growth factor receptor (EGFR), vascular Endothelial Growth Factor Receptor 3 (VEGFR3, also known as FLT4), KIT proto-oncogene (KIT), Fms-like tyrosine kinase 3 (FLT3), MET proto-oncogene (MET), vascular Endothelial Growth Factor Receptor (VEGFR1, also known as FLT1), mammalian target of rapamycin (mTOR), platelet-derived growth factor receptor alpha (PDGFRA), and platelet-derived growth factor receptor beta (PDGFRB).

Construction of the lncRNA–miRNA–mRNA Regulatory Network

The DIANA online tools[6] were employed to explore the miRNAs binding to lncRNA. We employed three miRNA databases, including miRDB[7], miRTarBase[8], and TargetScan[9], to predict the target genes of miRNAs. To predict the expression correlation between lncRNAs and miRNAs, the threshold was set at 0.9. Subsequently, the lncRNA-miRNA-mRNA regulatory network was mapped by the Cytoscape (version 3.7.0)[10] to better understand the connections.

Functional Annotation and Pathway Analysis of the Target mRNAs

To further explore the functional annotation and pathway analysis of the target mRNAs, the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed by using the "clusterProfiler" R package with a $p < 0.05$, and FDR < 0.05.

Identification of Potential Small Molecule Drugs

Connectivity Map (CMap)[11] is a collection of genome-wide transcriptional expression data from cultured human cells treated with bioactive small molecules and analyzed by corresponding matching algorithms to investigate the relationship between drug and gene expression changes and phenotypes (Lamb et al., 2006). We uploaded up- and down-regulated target genes from the lncRNA-miRNA-mRNA network to CMap. A connectivity score ranging from -1 to 1 was used to reflect the degree of closeness between the expression spectrums. The drugs with negative scores were potential therapeutic molecules. Moreover, these candidate drugs were investigated in the Pubchem database[12].

Gene Expression Profiling Interactive Analysis (GEPIA)

Gene Expression Profiling Interactive Analysis (GEPIA)[13] is a website for large-scale expression analysis and interactive analysis that has been used to compare the expression of signature lncRNAs (Tang Z. et al., 2019).

Patients and Samples

Blood samples of PC patients were collected from the Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China) between May 2019 and July 2019. Patients were eligible if they didn't receive any preoperative radiation and chemotherapy and the postoperative pathology was officially diagnosed as pancreatic adenocarcinoma. Exclusion criteria are as follows: (1) patients with history of previous cancer; (2) patients with multiple tumors or PC is not a primary lesion; and (3) patients with co-morbidities of the blood system. Finally, 30 of the 37 blood samples were eligible for further study. And, 10 blood samples of healthy donors were collected as a control group.

The serum specimen was separated at 3,000 rpm for 10 min from the venous blood. All the serum samples were stored at −80°C. Ethical approval for the use of human samples was obtained from the Tongji Hospital Research Ethical Committee.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from serum samples and cells by the TRIzol reagent (Life Technologies, Thermo Fisher Scientific, United States). The complementary DNA was synthesized with the PrimeScript^{TM} RT Master Mix (Takara Bio Inc, Dalian, China) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed using a SYBR Green PCR kit (Thermo Fisher Scientific) following the standard protocol. And GAPDH served

[6]http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex

[7]http://mirdb.org/miRDB/

[8]http://mirtarbase.mbc.nctu.edu.tw/

[9]http://targetscan.org/

[10]https://cytoscape.org/

[11]https://portals.broadinstitute.org/cmap/

[12]https://pubchem.ncbi.nlm.nih.gov/

[13]http://gepia2.cancer-pku.cn/

as the internal control. The forward primer for LINC01559 was 5′-GTCCTGCAGAACTCCCTCTT-3′, the reverse primer for LINC01559 was 5′-AGTCCTGGAGCTGCAGAAAT-3′. The forward primer for AC245041.2 was 5′-TTGCCCCCATCTTTGCCATTCC-3′, the reverse primer for AC245041.2 was 5′- TTGACCCATCTTTCCTCCCCAC-3′. The forward primer for AC005332.6 was 5′-AAGACAGCACG GTGTTAAAAAG-3′, the reverse primer for AC005332.6 was 5′-TTGAATCCAGGAGGCGGAAG-3′. The forward primer for GAPDH was 5′-GGAGCGAGATCCCTCCAAAAT-3′, the reverse primer for GAPDH was 5′-GGCTGTTGTCATACTT CTCATGG-3′. The relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

Cell Culture and Transfection

Human PC cell lines PANC-1 and SW1990 were obtained from the American Type Culture Collection (Manassas, VA, United States). These cells were maintained in the Dulbecco's modified Eagle medium (Gibco, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 units/mL of penicillin, and 100 mg/mL of streptomycin (Sigma-Aldrich, St. Louis, MO, United States). All cell lines were authenticated, mycoplasma-free and cultured at 37°C in a humidified incubator containing 5% CO_2.

LINC01559 si-1, LINC01559 si-2 and si-NC were purchased from the DesignGene Biotechnology (Shanghai, China) and transfected into PC cells using the Lipotransfectamine 3000 (Thermo Fisher Scientific). The lentiviral vector containing the tandem-labeled GFP-mRFP-LC3 reporter were also constructed by the DesignGene Biotechnology (Shanghai, China), and transfection was carried out according to the manufacturer's specification.

Western Blot Analysis

Western blotting assay was performed to detect the expression of LC3B, p62, and GAPDH as previously described (Tian et al., 2018). The antibodies of LC3B (#2775, 1:200), p62/SQSTM1 (#5114, 1:500), cleaved caspase-3 (#9661, 1:1,000), cleaved PARP (#9544, 1:1,000), and GAPDH (#5174, 1:1,000) were purchased from the Cell Signaling Technology (CST, Danvers, MA, United States). And the intensity of bands was estimated by the Image J2X (National Institute of Mental Health, Bethesda, MD, United States). All experiments were repeated three times.

Transwell Assay

For migration assay, 1×10^5/mL PC cells were suspended into the upper transwell chamber of 24-well transwell plates (8 μm pore size; Corning) containing 200 μL serum-free medium, while the lower chambers were supplied with a 500 μL complete culture medium. After 48 h co-culture, the cells on the lower surface of membrane were fixed in 4% paraformaldehyde and stained with crystal violet solution. The stained cells were then counted under a Nikon light microscope (Nikon, Japan). For invasion assay, the upper transwell chambers were coated with 60 μL Matrigel matrix gel (BD Biosciences, United States). The other operations were the same as the transwell migration experiment. All experiments were repeated three times.

Wound Healing Assay

Indicated PC cells (2×10^5 cells/well) were seeded in 6-well plates to grow to 90% confluence, and then we scratched the wound with a 200 μL pipette tip across the center of the well. After washing three times with PBS, the cells were incubated in a serum-free medium at 37°C with 5% CO_2. Wound healing was observed with the optical microscope (Nikon, Japan) at 0 and 24 h, respectively. All experiments were repeated three times.

In vitro Drug Cytotoxic and Cell Proliferation Assay

A Cell Counting Kit-8 (Dojindo Laboratories Co. Ltd, Kumamoto, Japan) assay was used to evaluate cell viability. Briefly, PANC-1 and SW1990 cells were seeded in 96-well plates at a density of 2×10^3 cells per well. Each group had triplicates (n = 3). Cells were treated with gemcitabine at indicated concentration after 48 h. At the indicated time point, 10 μL of CCK-8 solution was added into each well and cells were incubated for 2 h at 37°C with 5% CO_2. Then, the absorbance was measured at 450 nm with a plate reader (Bio-Tek Elx 800, United States). To assess cell proliferation, PC cells (1×10^3 cells/well) were placed into 96-well plates and measured at 0, 24, 48, 72, 96. All experiments were repeated three times.

TABLE 1 | Clinical information of pancreatic cancer patients in the training, validation, and entire cohort.

Variable	Entire cohort (n = 177)	Training cohort (n = 107)	Validation cohort (n = 70)	p-value
Age				
≤ 65	93 (52.54%)	53 (49.53%)	40 (57.14%)	0.4023
> 65	84 (47.46%)	54 (50.47%)	30 (42.86%)	
Gender				
Female	80 (45.2%)	52 (48.6%)	28 (40%)	0.3324
Male	97 (54.8%)	55 (51.4%)	42 (60%)	
Grade				
G1–2	125 (70.62%)	76 (71.03%)	49 (70%)	0.4866
G3–4	50 (28.25%)	29 (27.1%)	21 (30%)	
GX	2 (1.13%)	2 (1.87%)	0 (0%)	
Stage				
I–II	167 (94.35%)	102 (95.33%)	65 (92.86%)	1
III–IV	7 (3.95%)	4 (3.74%)	3 (4.29%)	
Unknown	3 (1.69%)	1 (0.93%)	2 (2.86%)	
T stage				
T1–2	31 (17.51%)	22 (20.56%)	9 (12.86%)	0.3011
T3–4	144 (81.36%)	85 (79.44%)	59 (84.29%)	
Unknown	2 (1.13%)	0 (0%)	2 (2.86%)	
M stage				
M0	79 (44.63%)	48 (44.86%)	31 (44.29%)	0.9106
M1	4 (2.26%)	2 (1.87%)	2 (2.86%)	
MX	94 (53.11%)	57 (53.27%)	37 (52.86%)	
N stage				
N0	49 (27.68%)	33 (30.84%)	16 (22.86%)	0.1963
N1	123 (69.49%)	72 (67.29%)	51 (72.86%)	
NX	5 (2.82%)	2 (1.87%)	3 (4.29%)	

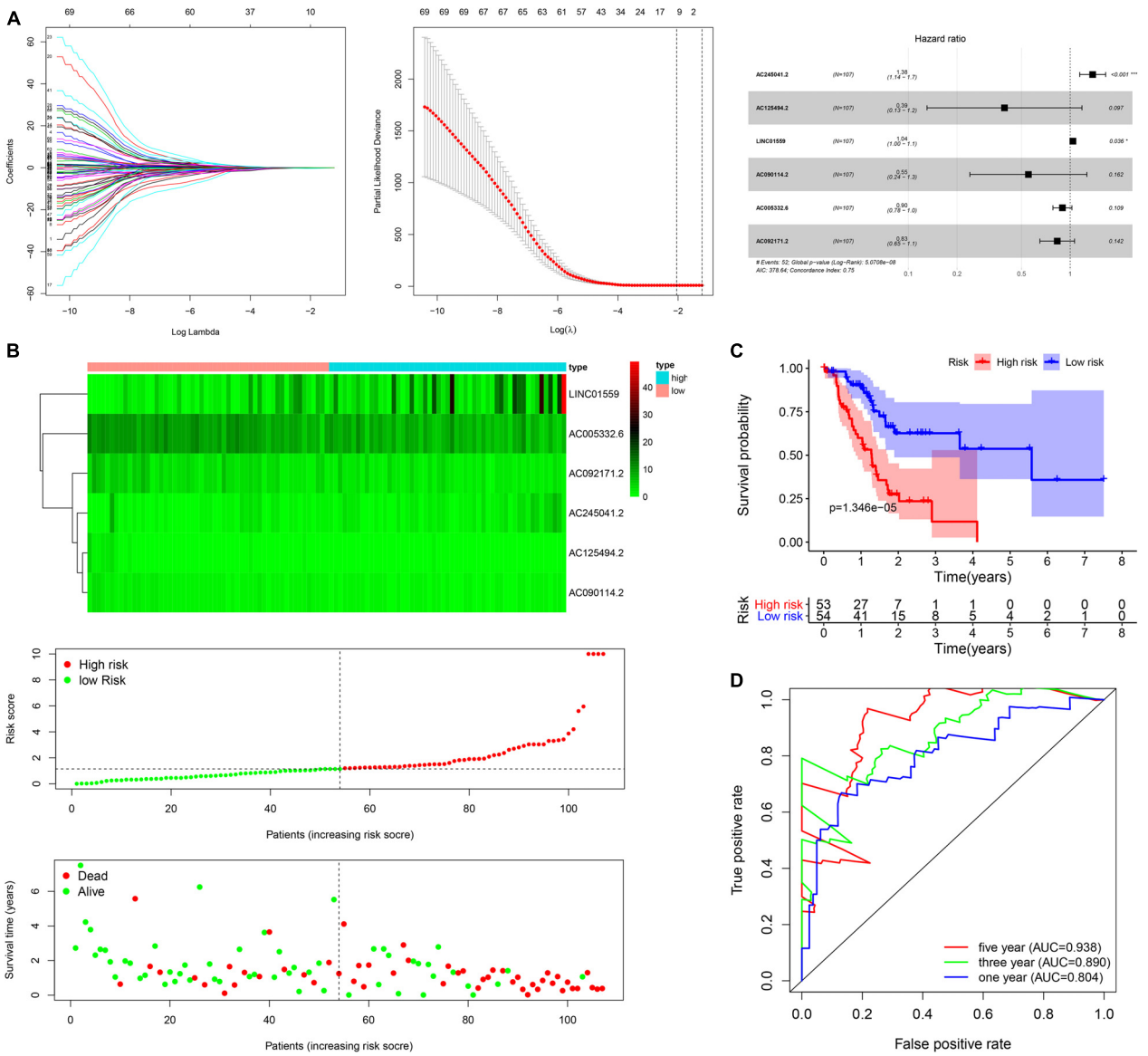


FIGURE 1 | Construction of the autophagy related lncRNAs signature in training cohort. **(A)** The procedure of the construction of the SRGs signature, including univariate Cox regression analysis (left), LASSO penalized Cox regression analysis (middle) and multivariate Cox regression analysis (right). **(B)** Correlation between the prognostic signature and the overall survival of patients. The distribution of selected ARlncRNAs (upper), risk score (middle) and survival time (below). **(C)** Kaplan-Meier survival analysis of high-risk and low-risk risk group. **(D)** Receiver operating characteristic (ROC) curves for 1, 3, and 5 years survival (*$p < 0.05$; ***$p < 0.001$; ns, not significant).

Cell Apoptosis Assay

The percentage of apoptotic cells was analyzed by the PE Annexin V Apoptosis Detection kit (BD Pharmingen). Briefly, 1×10^5/mL cells indicated PC cells were seeded into six-well culture plates. After 48 h of relevant treatment, the cells were harvested using trypsin without EDTA and washed twice with binding buffer. Finally, the cells were resuspended in 100 μL of binding buffer containing 5 μL Annexin V-PE and 5 μL 7-ADD for 15 min in the dark at room temperature, and the apoptosis was detected by flow cytometry (BD Biosciences, Franklin Lakes, NJ, United States). All experiments were repeated three times.

Colony Formation Assay

Indicated PC cells (1×10^3 cells/well) were seeded into 6-well plates and cultured in completed medium at 37°C with 5% CO_2 for 2 weeks. Then, the cells were cleaned, fixed and dyed, and finally photographed. All experiments were repeated three times.

Immunofluorescence and Confocal Microscopy

Cells transfected with GFP-mRFP-LC3B were grown on glass coverslips. Following the indicated treatments,

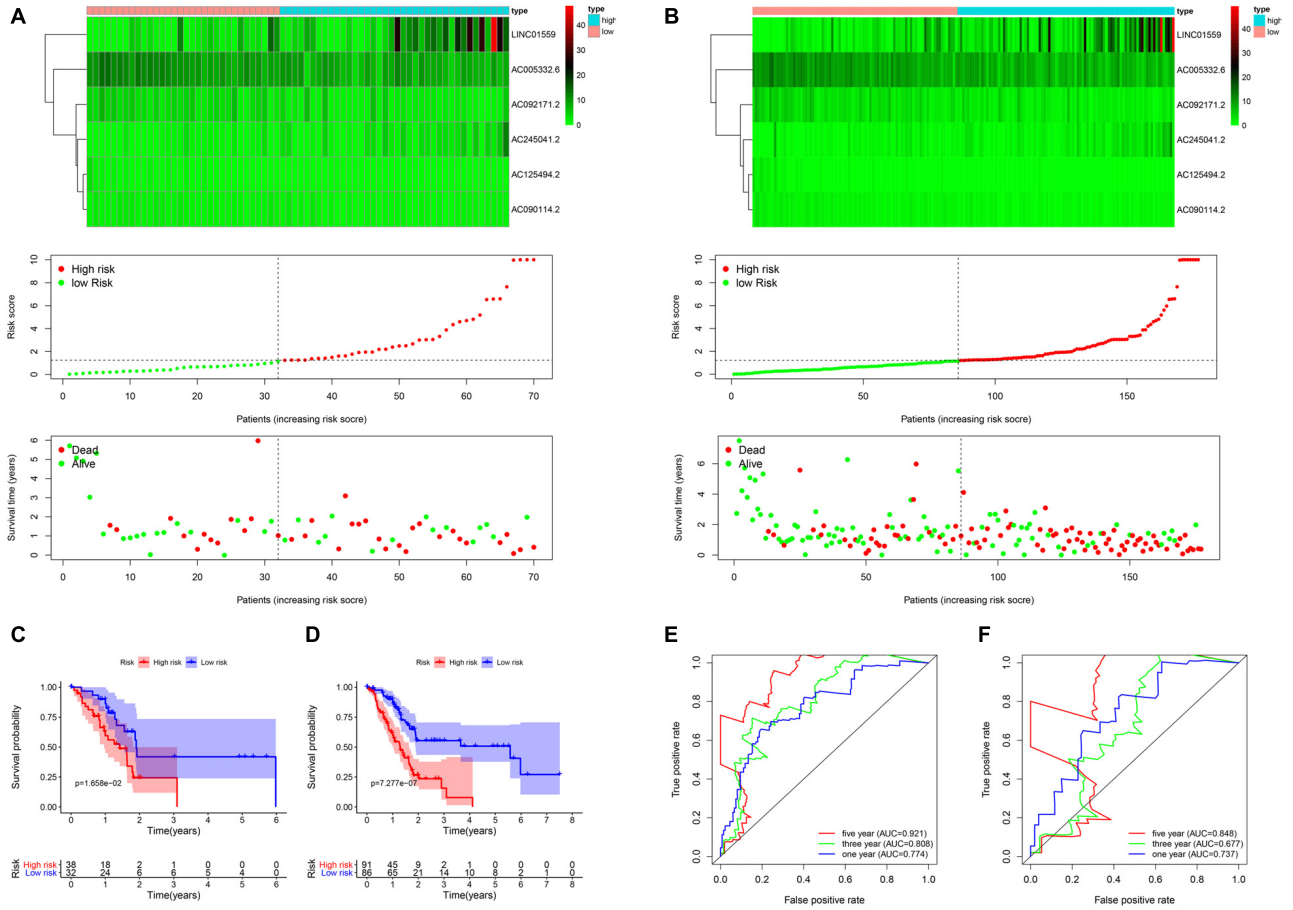


FIGURE 2 | The validation of the risk model in the validation cohort and entire cohort. **(A,B)** Correlation between the prognostic signature and the overall survival of patients in the validation set **(A)** and entire set **(B)**. The autophagy related lncRNAs levels (upper), risk scores (middle), survival time (below). **(C,D)** Kaplan–Meier curves of overall survival in the training cohort **(C)** and the validation cohort **(D)** stratified by risk scores. **(E,F)** Receiver operating characteristic (ROC) curve analysis for the survival prediction model in the training cohort **(E)** and the validation cohort **(F)**.

cells were fixed with 4% formaldehyde for 30 min and photographed under a confocal microscope (Carl Zeiss, Germany, LSM710).

Statistical Analysis

All statistical analysis and plotting were performed in the R language (Version 3.6.2). Univariate and multivariate Cox regression analyses were used to identify independent prognostic factors for PC. And $p < 0.05$ was considered statistically significant.

RESULTS

Identification of Autophagy-Related lncRNAs (ARlncRNAs)

A total of 13,482 lncRNAs was extracted from the TCGA dataset, 825 of which were identified as ARlncRNAs by the Pearson correlation analysis ($|R| > 0.5, p < 0.01$).

Establishment and Verification of a Prognostic ARlncRNAs Signature

Firstly, all patients were divided into training cohort ($n = 107$) and validation cohort ($n = 70$) (**Table 1**). Then, we employed the LASSO penalized cox regression analysis by the training cohort and found 10 more representative ARlncRNAs: AC245041.2, AL354892.2, FLVCR1.DT, AC125494.2, AL162274.2, LINC01559, AC090114.2, SH3PXD2A.AS1, AC005332.6, and AC092171.2 (**Figure 1A**). Moreover, the stepwise multivariate Cox regression was utilized to establish a predictive signature for PC patients in the training cohort with a risk score = (0.319702425 × expression level of AC245041.2) + (−0.934877496 × expression level of AC125494.2) + (0.038664123 × expression level of LINC01559) + (−0.594425726 × expression level of AC090114.2) + (−0.110425977 × expression level of AC005332.6) + (−0.184537572 × expression level of AC092171.2) (**Figure 1A**).

Next, the patients in the training cohort were divided into high-risk group and low-risk group based on the median risk score. **Figure 1B** showed the distribution of prognostic

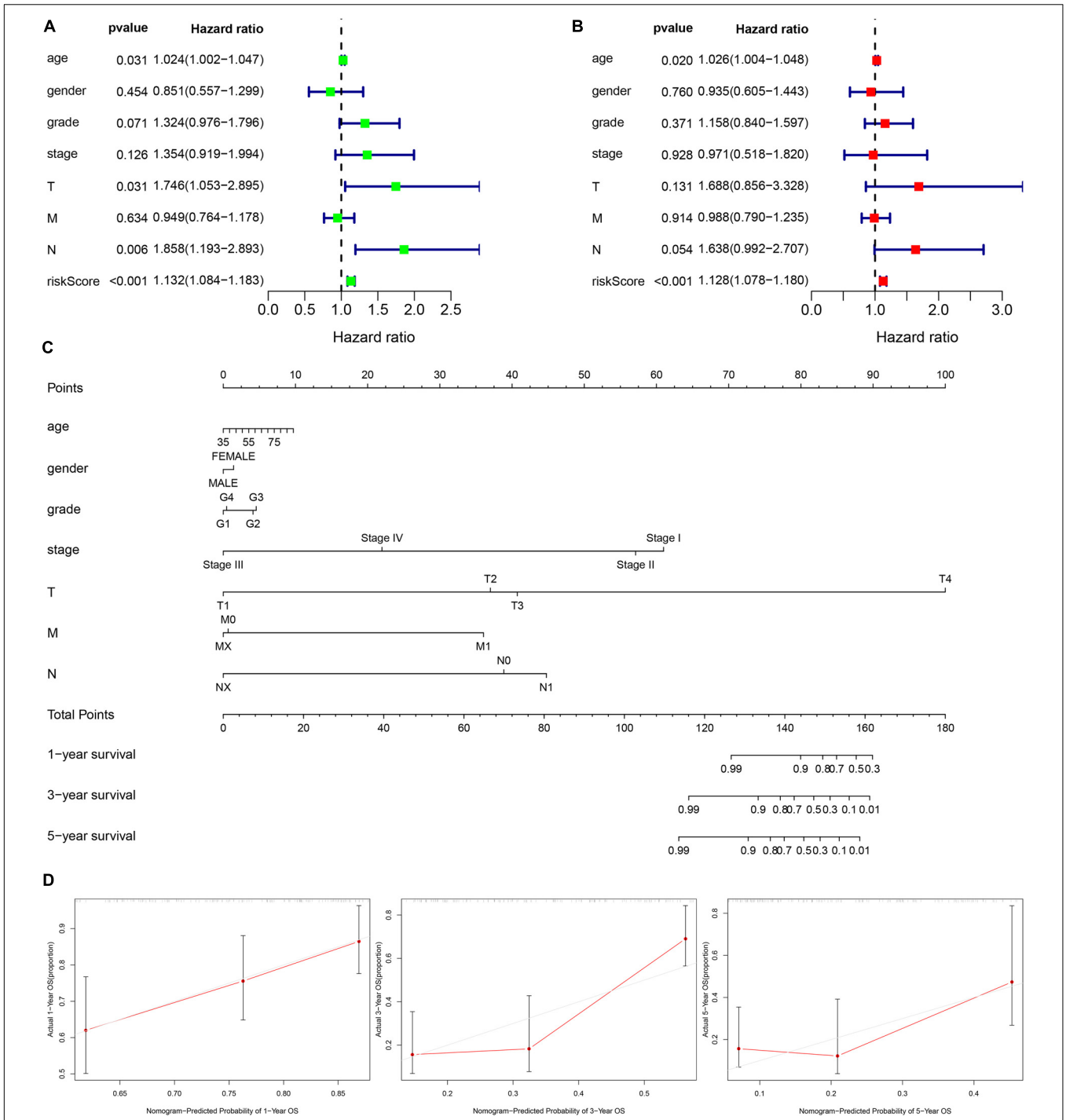


FIGURE 3 | Establishment of a nomogram based on clinical characteristics and the autophagy related lncRNAs signature. **(A,B)** Univariate Cox regression analysis **(A)** and Multivariate Cox regression analysis **(B)** of clinical features and the signature. **(C)** The nomogram consists of clinical characteristics and prognostic signature. **(D)** The nomogram calibration curve is used to predict 1, 3, and 5 years survival rates.

signature, survival outcomes of PC patients in different groups, and the expression profiles of the selected lncRNAs. Notably, the Kaplan-Meier survival analysis in the training cohort revealed that the survival time of PC patients was significantly longer in the low-risk group than the high-risk group (**Figure 1C**). As shown in **Figure 1D**, the area under the ROC (AUC) for 1, 3, and 5 years of the survival were 0.938, 0.890, and 0.804 in the training cohort, suggesting that the signature exerted a certain potential property for prognostic prediction in PC patients.

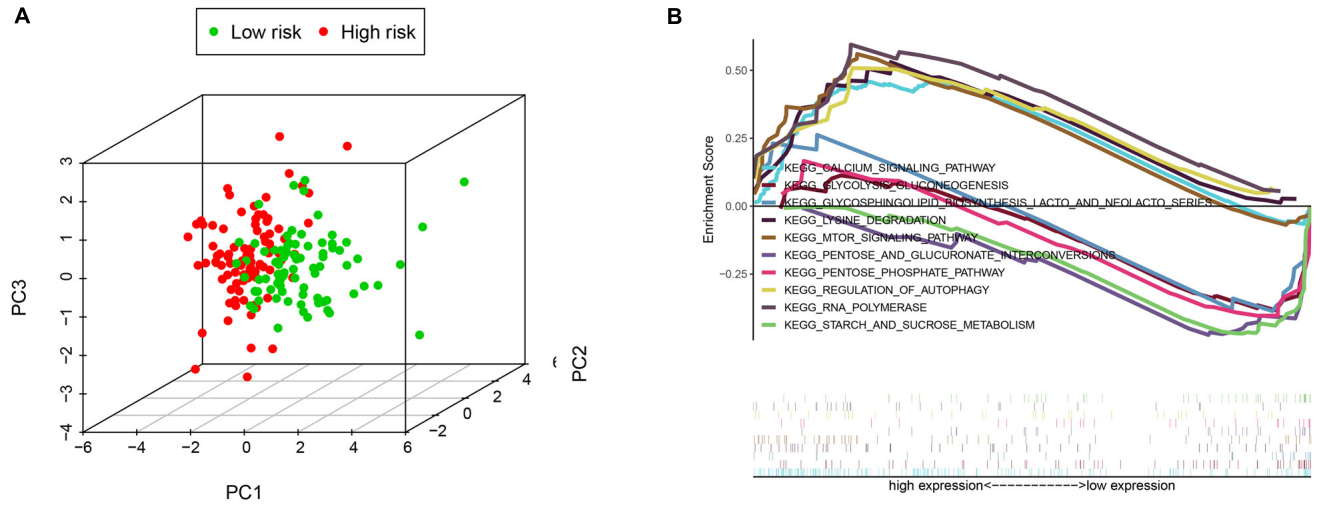


FIGURE 4 | The low-risk and high-risk groups displayed different autophagy statuses. **(A)** Principal components analysis between low- and high-risk groups on the basis of the autophagy-related gene sets. **(B)** Gene set enrichment analysis (GSEA) of the two groups.

To verify the accuracy of the signature, we analyzed its prognostic value in the validation cohort and entire cohort. LncRNAs expression profiles, risk distribution, and survival rate in the validation cohort and entire cohort were shown in **Figures 2A,B**. Similar to the results in the training cohort, the Kaplan-Meier (K-M) survival analysis in both the validation cohort and entire cohort indicated that the survival outcome of PC patients was better in low-risk group than in high-risk group (**Figures 2C,D**). The AUC at 1, 3, and 5 years were and 0.848, 0.677, 0.737, and 0.921, 0.808, 0.774 in the validation cohort and entire cohort, respectively (**Figures 2E,F**).

Furthermore, the univariate and multivariate Cox regression analysis were employed to confirm the independent prognostic role of the signature for PC patients in the entire cohort (**Figures 3A,B**). Another independent prognostic factor was age. Moreover, a nomogram based on the signature risk score and clinical features was constructed and the calibration curve for 1, 3, and 5 years of the nomogram showed a great predictive power of the prognostic signature (**Figures 3C,D**).

Functional Analysis of the Signature

PCA was employed to demonstrate the significant distribution difference between low- and high-risk groups based on the risk scores (**Figure 4A**). Then, GSEA was implemented to explore the significant enriched pathways between the two groups. As shown in **Figure 4B**, the top five up-regulated and down-regulated KEGG pathways were the "mTOR signaling pathway," "calcium signaling pathway," "regulation of autophagy," "RNA polymerase," "lysine degradation," and "pentose phosphate pathway," "starch and sucrose metabolism," "glycolysis gluconeogenesis," "glycosphingolipid biosynthesis lacto and neolacto-series," and "pentose and glucuronate interconversions," respectively. These results indicated that the high-risk score was significantly associated with autophagy regulation and several signaling pathways may participate in the process.

The Relationship Between the Signature and the Clinical Features in PC

To investigate the clinical utility of the signature, we explored the relationship of the signature with clinical features. We found that the high-risk score was significantly correlated with tumor grade, AJCC stage, N stage, T stage, and survival status (**Figure 5A**).

The Relationship of the Signature and Immune Cell Infiltration in PC Tissues

To investigate the relationship between the prognostic signature and immune cell infiltration. Pearson correlation analysis showed that the signature score was significantly correlated with the infiltration of activated dendritic cells (cor = 0.152, p = 0.043), plasma cells (cor = −0.155, p = 0.040), CD8 T cells (cor = −0.193, p = 0.010), M1 macrophages (cor = 0.200, p = 0.008), and neutrophils (cor = 0.152, p = 0.043) (**Figure 5B**).

Predicting Efficacy of Immunotherapy and Targeted Therapy With the Signature

The tumor mutation burden (TMB) has been shown to be related to the clinical efficacy of immunotherapy (Samstein et al., 2019). To explore the value of our signature for predicting the efficacy of immunotherapy in PC, we assessed the TMB of PC patients in the high-risk and low-risk groups. We found that TMB of PC patients in the high-risk group was higher than that in the low-risk group, which implied that immunotherapy may be a potentially effective treatment to those PC patients with high-risk scores (**Figure 5C**).

Furthermore, we analyzed the correlation of the signature score with the therapy-related targets. Pearson's correlation analysis showed that the risk score was significantly associated

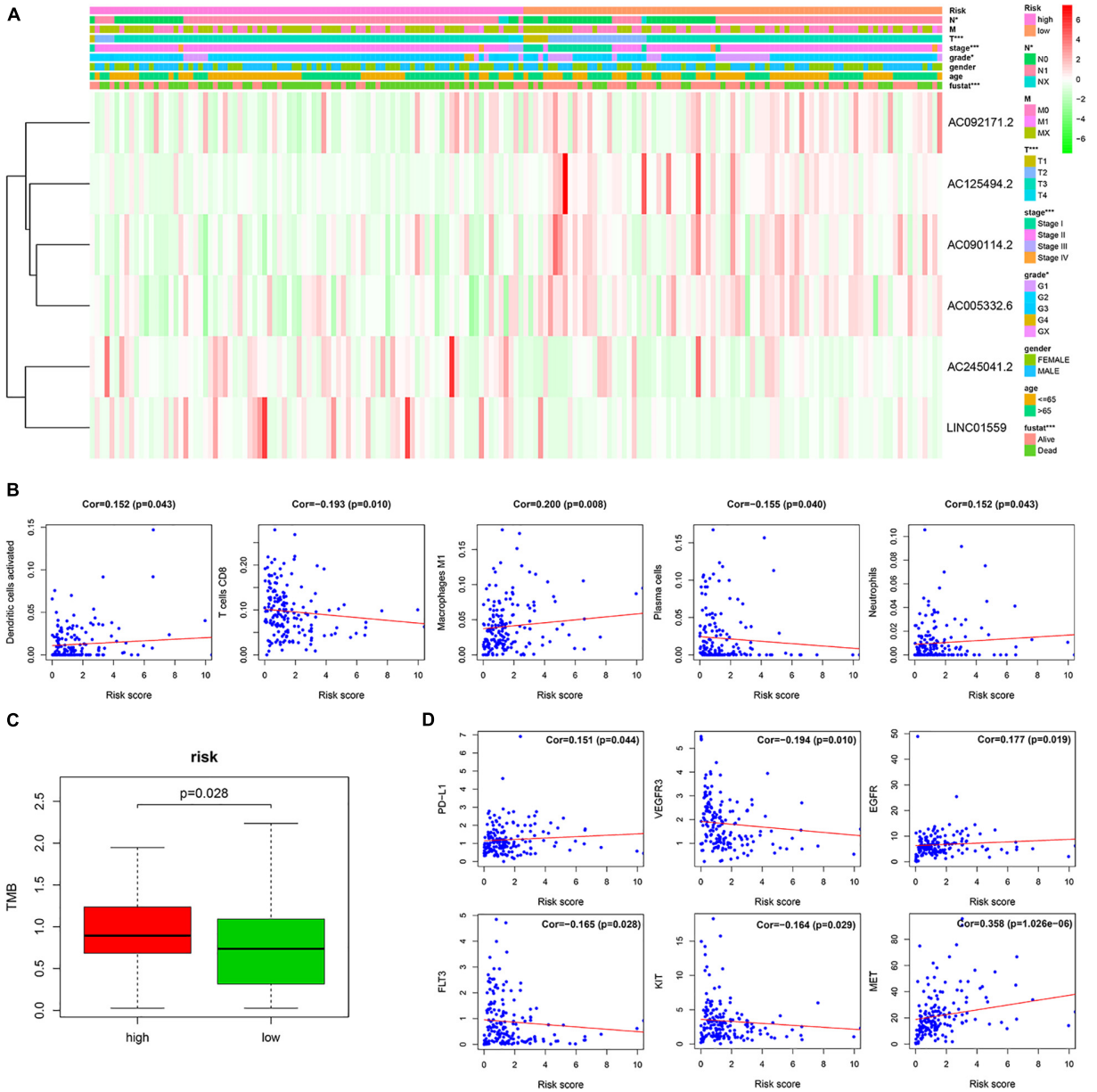


FIGURE 5 | The correlation of the risk score with the clinical traits, immune cells and therapy targets. **(A)** Correlation of risk group and clinical traits. **(B)** The correlation between the risk score and immune cells. **(C)** The TMB of PC patients in the high-risk and low-risk groups. **(D)** The correlation between the risk score and therapy targets (*$p < 0.05$; ***$p < 0.001$; ns, not significant).

with PD-L1 (cor = 0.151, $p = 0.044$), VEGFR3 (cor = −0.194, p = 0.010), EGFR (cor = 0.177, $p = 0.019$), FLT3 (cor = −0.165, $p = 0.028$), KIT (cor = −0.164, $p = 0.029$), and MET (cor = 0.358, $p = 1.026e{-}06$) (**Figure 5D**).

Construction of the lncRNA–miRNA–mRNA Regulatory Network

LncRNAs could interact with miRNAs to modulate mRNA expression, thereby modulating the biological characteristics of malignant tumors. To explore the regulation of these selected LncRNAs, we constructed a regulatory network consisting of six lncRNAs, 107 miRNAs, and 209 mRNAs (**Figure 6**).

Functional Analysis of the Regulatory Network

To better understand the function of the regulatory network, the "clusterProfiler" R package was employed to conduct a KEGG pathway and GO enrichment analysis. As shown in **Figure 7**, these genes in the regulatory network are

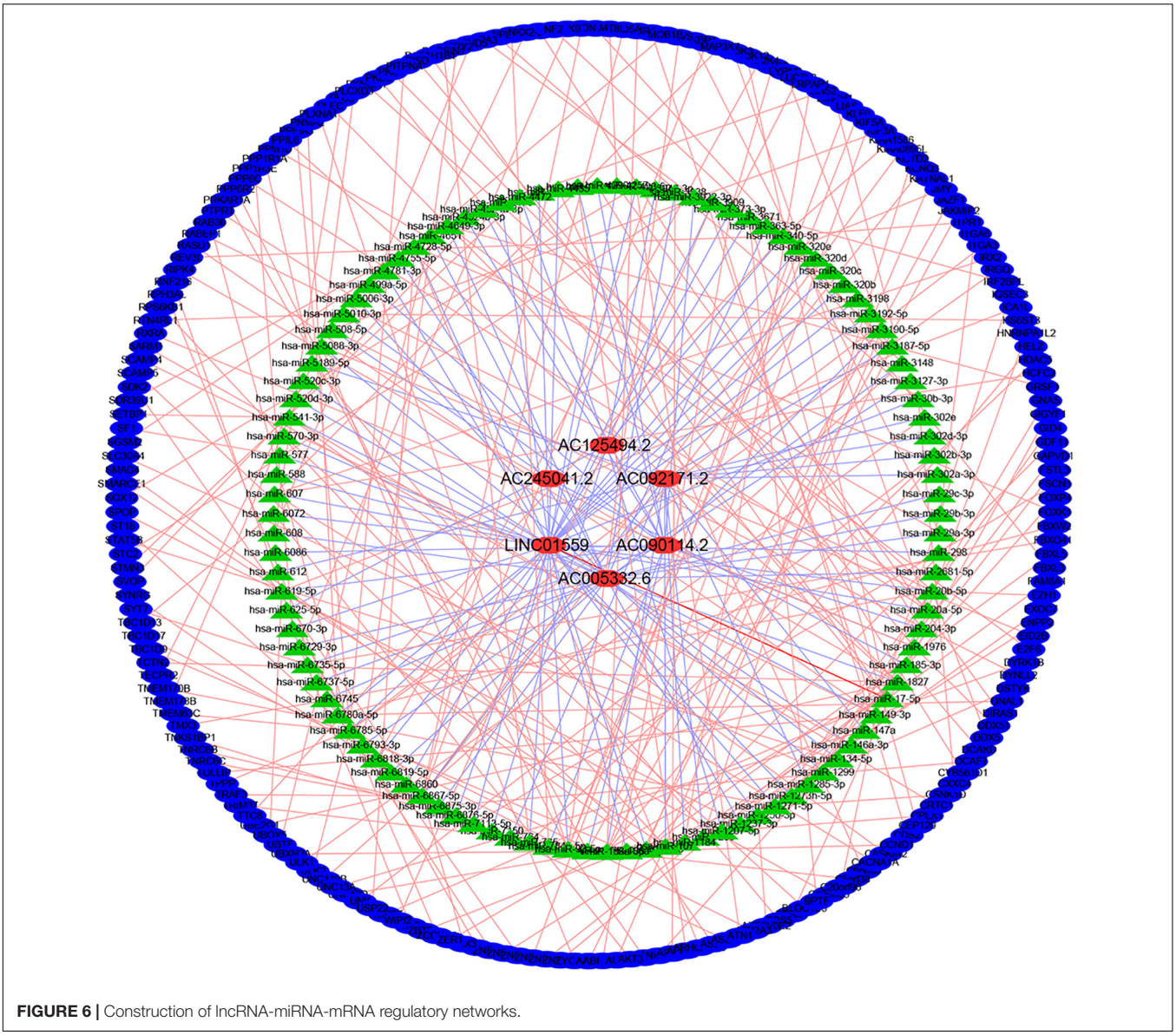


FIGURE 6 | Construction of lncRNA-miRNA-mRNA regulatory networks.

enriched in many cellular components (CC) and molecular functions (MF). The most significantly enriched molecular functions included "protein serine/threonine kinase activity," "Rab GTPase binding," and "protein serine/threonine/tyrosine kinase activity," In terms of KEGG pathway, the main significant pathways included "autophagy," "small cell lung cancer," "Toll-like receptor signaling pathway," "pancreatic cancer," "ErbB signaling pathway," "colorectal cancer," "endocrine resistance," and "hedgehog signaling pathway." These results indicated that the regulatory network may contribute to therapeutic resistance of PC through multiple signal pathways.

Potential Small Molecule Drugs Screening

To screen small molecule drugs, 209 selected mRNA were further analyzed in the Connectivity Map (CMap). The top six most significant potential small molecule drugs were listed in **Figure 8A**, including vorinostat ($C_{14}H_{20}N_2O_3$), trichostatin A ($C_{17}H_{22}N_2O_3$), sirolimus ($C_{51}H_{79}NO_{13}$), phthalylsulfathiazole ($C_{17}H_{13}N_3O_5S_2$), GW-8510 ($C_{21}H_{15}N_5O_3S_2$), and daunorubicin ($C_{27}H_{29}NO_{10}$). And the 2D chemical structures of these potential agents were shown in **Figure 8B**.

Serum LINC01559 Served as a Diagnostic Biomarker

Firstly, we evaluated the expression profiles and prognostic performance of these six lncRNAs. The expression level of AC245041.2, LINC01559, and AC005332.6 was significantly upregulated in PC than in normal tissues (**Figure 9A**). Moreover, the Kaplan-Meier survival analysis demonstrated the prognostic power of these six lncRNAs (**Figure 9B**). Then, qRT-PCR was

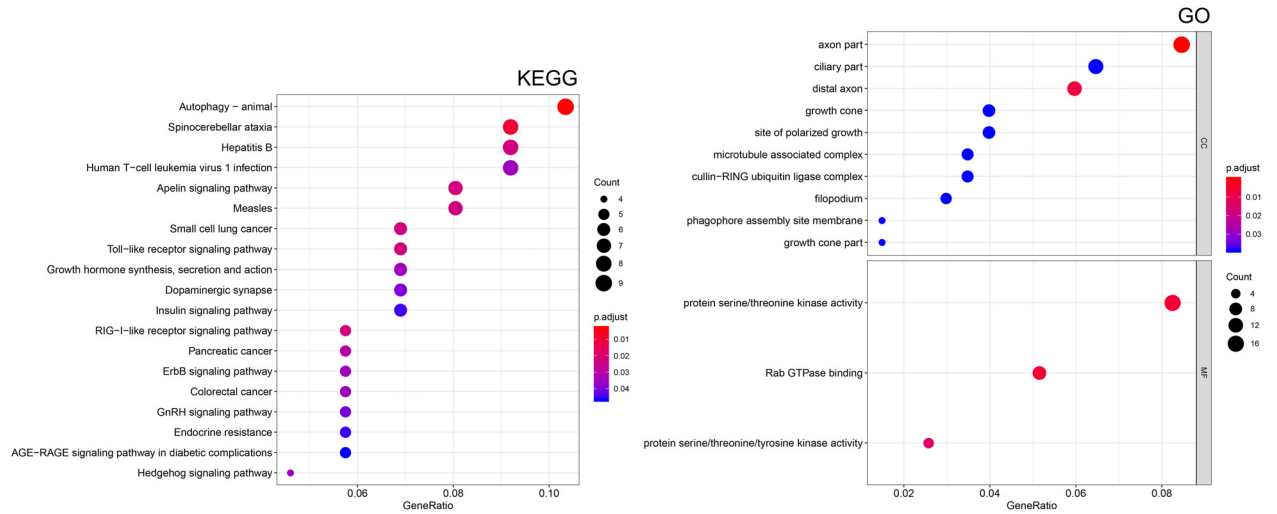


FIGURE 7 | Functional enrichment analysis of target mRNAs. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of mRNAs. Gene ontology (GO) enrichment analysis of the mRNAs. CC, cellular component; MF, molecular function.

applied to value the expression level of these lncRNAs in serum. Notably, only the expression of LINC01559 was markedly increased in the serum of PC patients, indicating that LINC01559 could serve as a diagnostic biomarker (**Figure 9C**).

Inhibition of LINC01559 Suppressed PC Cell Proliferation, Migration, and Invasion

We selected LINC01559 for further analysis. As shown in **Figure 10A**, we successfully silenced the expression level of LINC01559 in PC cells by si-LINC01559 transfection. Next, we explored the effect of silencing LINC01559 on the PC cells proliferation, migration, and invasion. CCK8 assay showed that the inhibition of LINC01559 led to a reduced viability in the PANC-1 and SW1990 cells (**Figure 10B**). Also, transwell assay was performed to demonstrate that the invasion and migration ability of PC cells were suppressed under LINC01559 depletion (**Figures 10C,D**). Furthermore, it was proved by wound healing assays that silencing LINC01559 obviously hindered the migration ability of PANC-1 and SW1990 cells (**Figure 10E**). These results suggested that knockdown of LINC01559 suppressed PC cell proliferation, migration, and invasion *in vitro*.

Inhibition of LINC01559 Suppressed PC Cell Autophagy and Promotes Apoptosis

The role of LINC01559 in autophagy and chemotherapeutic resistance was further explored. WB analysis were employed to show that PC cells transfected with si-LINC01559 exhibited decreased the expression of LC3I/LC3II but increased the p62 expression, indicating that autophagy was inhibited after LINC01559 depletion (**Figure 11A**). However, gemcitabine (10 μM) treatment induced autophagy in PC cells (**Figure 11A**). This observation was further confirmed by the tandem LC3B-RFP-GFP fluorescence microscopy assay. As shown in **Figure 11B**, gemcitabine increased the number of red-only LC3 puncta in PC cells, implying an increase of autophagic flux. Inhibition of LINC01559 reduced the number of red-only LC3 puncta in GFP-mRFP-LC3-transfected PC cells compared with the cells treated with gemcitabine. Besides cell viability assay, colony formation assay, cell apoptosis assay, and WB analysis of apoptotic markers were performed. CCK-8 results showed that the IC50 value for gemcitabine was significantly increased in LINC01559-silenced PC cells (**Figure 11C**). In contrast, knockdown of LINC01559 significantly induced the colony-forming capacity of PANC-1 and SW1990 cells (**Figure 11D**) and increased the gemcitabine-induced apoptosis rates (**Figure 11E**). And the protein level of cleaved caspase3 and PARP were increased in LINC01559-downregulated cells with or without gemcitabine (10 μM) treatment (**Figure 11F**). These results suggested that the inhibition of LINC01559 could suppress autophagy and stimulate apoptosis, which would ultimately lead to sensitize PC cells to gemcitabine.

DISCUSSION

PC is a solid tumor with a highly malignant behavior, which has become the fourth largest cancer causing cancer-related death in western countries, with a 5 years survival rate of less than 10% (Siegel et al., 2019). Accumulated evidence showed that autophagy got involved in tumor development and treatment resistance in PC (Piffoux et al., 2020). Thus, it is essential to screen autophagy-related molecular to identify effective prognostic biomarkers for PC. Recent great advances in genomics have provided opportunities for the identification of cancer prognostic biomarkers and potential molecular targets. Here, we were the first to construct a reliable prognostic signature based on autophagy-associated lncRNAs (ARlncRNAs) and confirmed the clinical utility in PC patients. Moreover, we preliminary explored

A **The top six most significant potential small molecule drugs based on CMAP.**

cmap name	mean	n	enrichment	p	specificity	% non-null
vorinostat	-0.606	12	-0.824	0.00000	0.0442	100
trichostatin A	-0.544	182	-0.587	0.00000	0.0693	86
sirolimus	-0.232	44	-0.327	0.00008	0.2534	56
phthalylsulfathiazole	-0.565	5	-0.759	0.00150	0.0201	100
GW-8510	-0.628	4	-0.83	0.00157	0.1679	100
daunorubicin	-0.603	4	-0.823	0.00185	0.0351	100

B

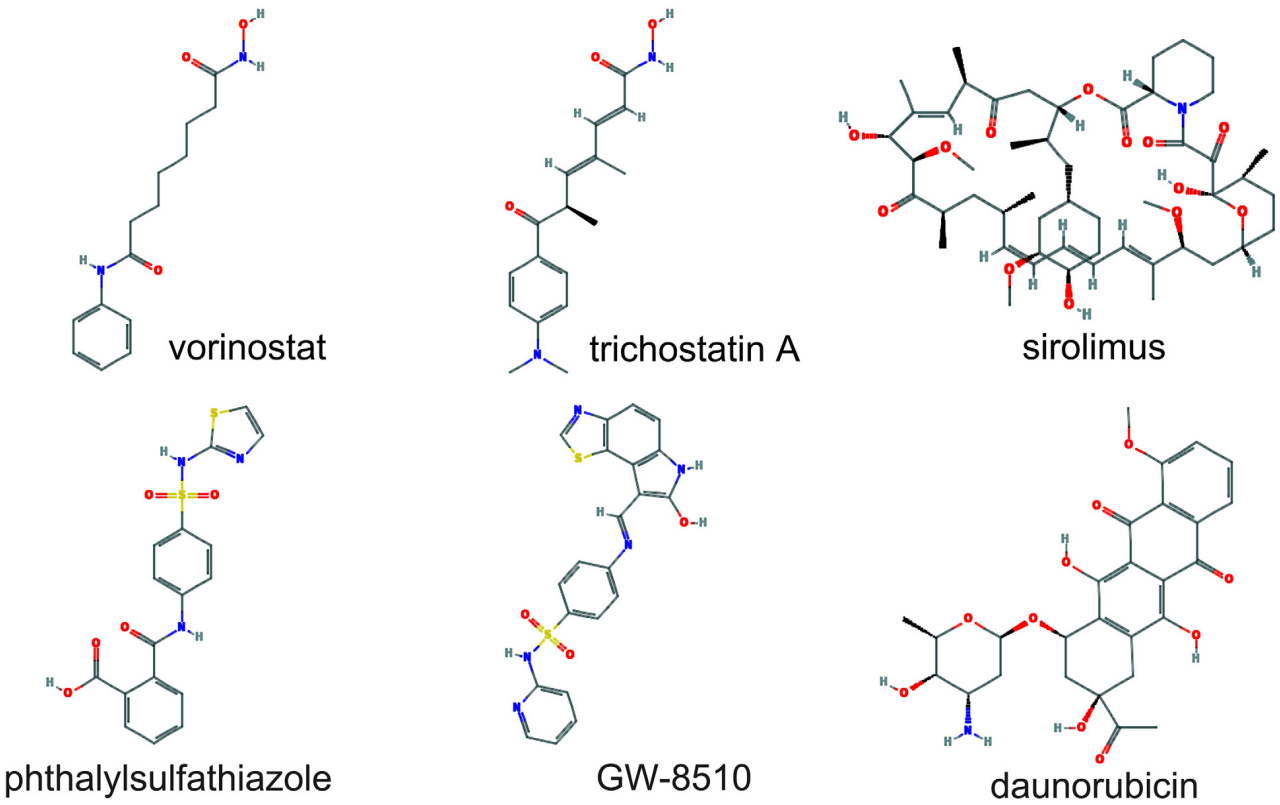


FIGURE 8 | Screening of six pancreatic cancer candidate small molecule drugs. **(A)** The top six most significant potential small molecule drugs based on cMAP. **(B)** The chemical structure depiction of the six candidate small molecule drugs for PC.

the carcinogenic role of LINC01559 in PC and found that the inhibition of LINC01559 might be a potential therapeutic strategy for improving sensitivity to gemcitabine in PC patients.

Firstly, we employed a Pearson correlation analysis to identify ARlncRNAs, and 826 lncRNAs were obtained. Then, these ARlncRNAs were screened to establish a six-ARlncRNAs signature in training cohort. Next, KM survival analysis and ROC analysis demonstrated the prognostic value of the signature in training cohort. And the similar results were also observed in both validation cohort and entire cohort. Furthermore, the independent prognostic role of the signature was confirmed by the univariate and multivariate cox regression analysis. Moreover, a nomogram indicated a great predictive power of the prognostic signature.

To further explore the clinical utility of the signature, we investigate the association of the signature with clinical features and found that high risk score was positive correlated with tumor grade, AJCC stage, N stage, and T stage. Immune cell infiltration analysis showed the risk score was negatively correlated with plasma cells and CD8 T cells. Moreover, the tumor mutation burden (TMB) of PC patients in the high-risk group was statistically higher than that in the low-risk group, indicating

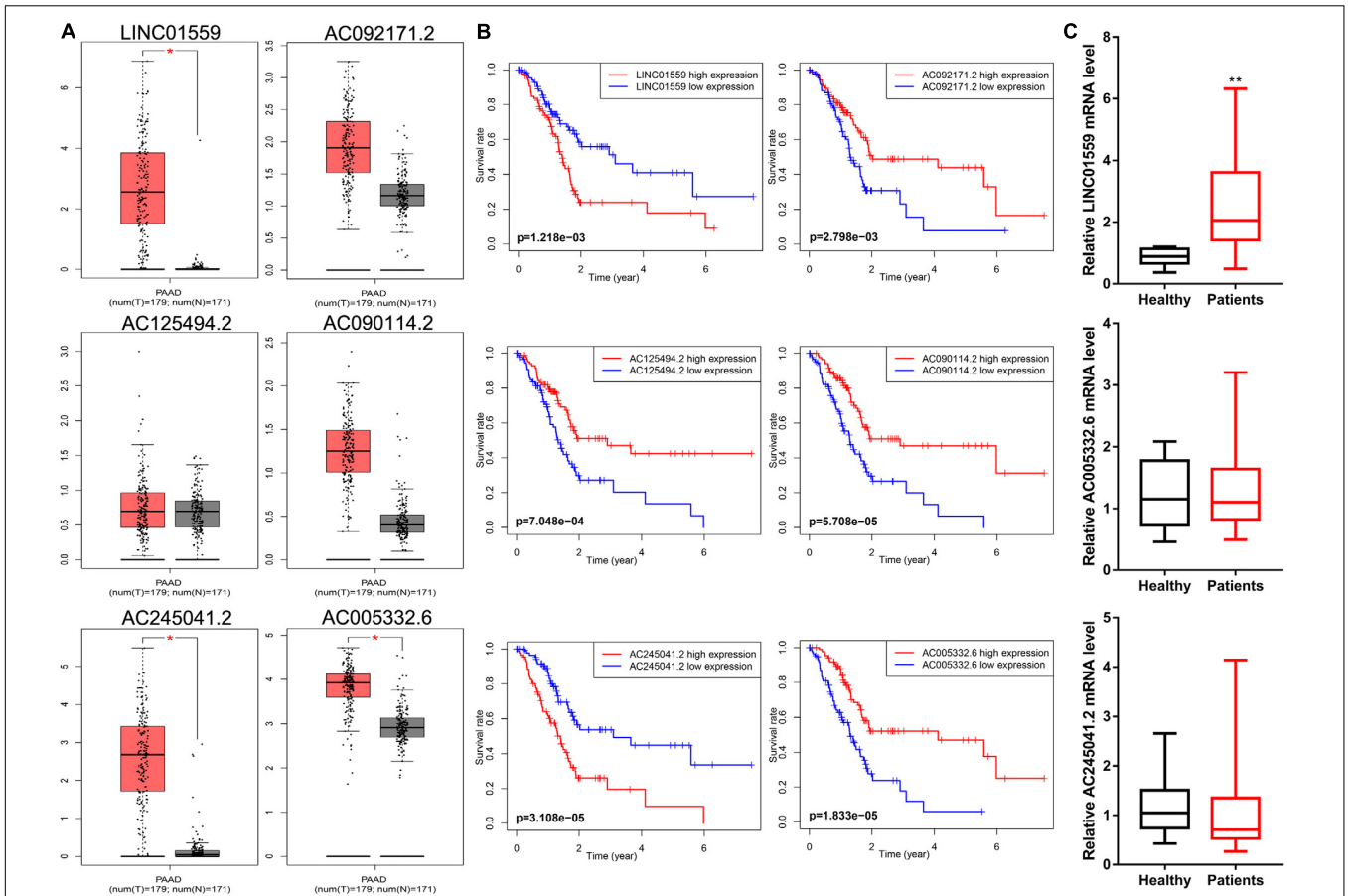


FIGURE 9 | LINC01559 serves as a diagnostic biomarker. **(A)** Comparison of differential expression of signature lncRNAs by GEPIA. **(B)** Kaplan-Meier survival curves for the signature lncRNAs. **(C)** qRT-PCR was conducted to detect the expression levels of LINC01559, AC245041.2, and AC005332.6 in serum (*$p < 0.05$; **$p < 0.01$; ns, not significant).

that immunotherapy may be a potentially effective option to those PC patients with high-risk scores. Next, correlation analysis showed that the signature scores were positively correlated with the expression of PD-L1, EGFR, and MET, implying that those PC patients with high-risk scores may be sensitive to these targeted chemotherapy drugs.

To better understand the potential biological mechanism of the involved ARlncRNAs, we constructed the lncRNA-miRNA-mRNA regulatory network. As indicated by KEGG pathway and GO enrichment analysis, the regulatory network may promote therapeutic resistance of PC. And several pathways, such as Toll-like receptor signaling pathway, ErbB signaling pathway, and hedgehog signaling pathway, may be involved in the process.

Most importantly, we identified six potential small molecule drugs from the network, including vorinostat, trichostatin-A, sirolimus, phthalylsulfathiazole, GW-8510, and daunorubicin. Vorinostat is a histone deacetylase (HDAC) inhibitor approved by FDA for the treatment of patients with refractory or relapsed cutaneous T cell lymphoma. Pre-clinical studies have demonstrated that vorinostat could induce apoptosis and inhibit tumor growth in human PC cell lines. And the combination of vorinostat and capecitabine with radiation in PC patients were well tolerated with antitumor activity in a phase I clinical trial (NCT00983268) (Chan et al., 2016). Trichostatin A (TSA), a natural derivative of dienohydroxamic acid, is a potent inducer of tumor cell growth arrest, differentiation, and apoptosis. Donadelli et al. (2003) demonstrated the antitumor value of TSA in human PC cell lines. And, combined therapy of gemcitabine and TSA exerted synergistic inhibition of PC cell growth (Donadelli et al., 2007). Sirolimus, also called rapamycin, is an immunosuppressive agent proved by FDA mainly for the prophylaxis of organ rejection in patients receiving renal transplants. However, as a derivative of sirolimus, everolimus exerts anti-angiogenic properties and is indicated as the first line therapy for pancreatic neuroendocrine tumor. And, clinical trials of everolimus in combination with other anticancer agents in PC patients is going on. Phthalylsulfathiazole is a broad-spectrum antimicrobial agent which is used in the treatment of dysentery, colitis, and gastroenteritis. It has not been reported that the agent shows anti-tumor effects. GW8510 is a synthetic cyclin-dependent kinase (CDK) inhibitor that could reverse tamoxifen resistance in breast cancer and gemcitabine resistance in lung squamous cell carcinoma through autophagy induction (Chen P. et al., 2018; Li et al., 2020). Daunorubicin

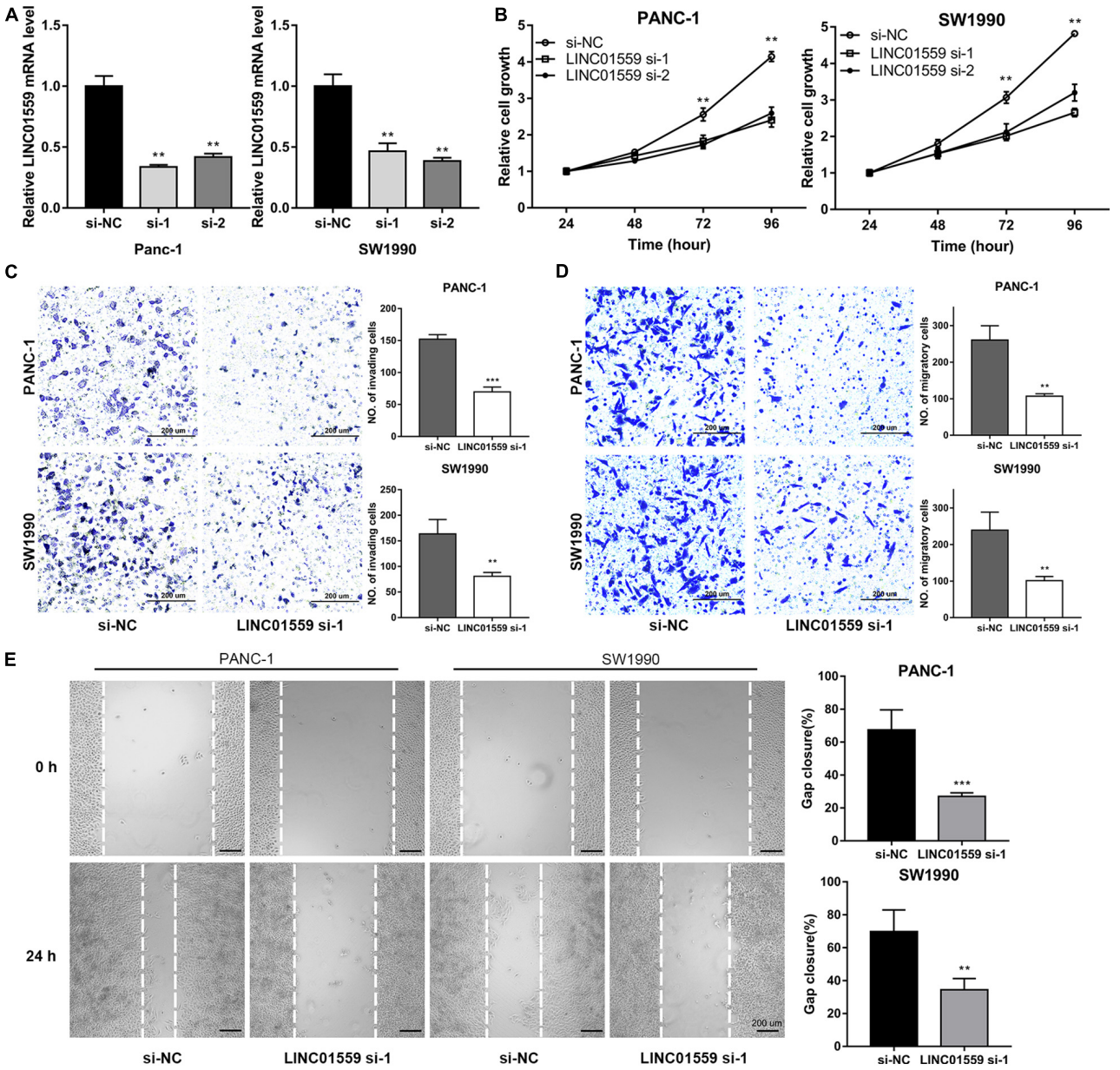


FIGURE 10 | Inhibition of LINC01559 suppresses PC cell proliferation, migration and invasion. **(A)** qRT-PCR was performed to measure the expression level of LINC01559 in PC cells transfected with si-NC or two siRNA targeting LINC01559. **(B)** CCK-8 analysis was used to examine the proliferation of PC cells. **(C,D)** Transwell assays were conducted to evaluate the effect of silencing LINC01559 on PC cell invasion ability **(C)** and migratory capacity **(D)**. Scale bar: 200 μm (200×). **(E)** Wound healing assays showing the capacity of indicated PC cell migration. Scale bar: 200 μm (40×) (**$p < 0.01$; ***$p < 0.001$; ns, not significant).

is the first generation of anthracyclines with antineoplastic activity and is indicated exclusively for the treatment of acute leukemia. Anthracycline drugs, including daunorubicin, doxorubicin, epirubicin, therarubicin, and aclacinomycin, are widely used in the treatment of hematologic malignancies and solid tumors. Taken together, these findings provide potential therapeutic options for patients with PC.

Among the six selected ARlncRNAs, LINC01559 was reported to be upregulated in renal cell carcinoma, gastric cancer, PC, and hepatocellular carcinoma (Chen B. et al., 2018; Chen et al., 2020; Dong et al., 2020; Lou et al., 2020; Wang et al., 2020). Moreover, we found that the expression of LINC01559 was significantly increased in both serum and tumor tissues of PC patients, indicating that LINC01559 could serve as a diagnostic biomarker. Thus, we chose LINC01559 for further analysis. Functional analysis showed inhibition of LINC01559 suppressed proliferation, migration, and invasion in PANC-1 and SW1990 cells. The results were similar to Lou et al. (2020) and Chen et al. (2020) in AsPC-1, BXPC-3, MIA PaCa-2 cells. Interestingly, Dong et al. (2020) reported that LINC01559

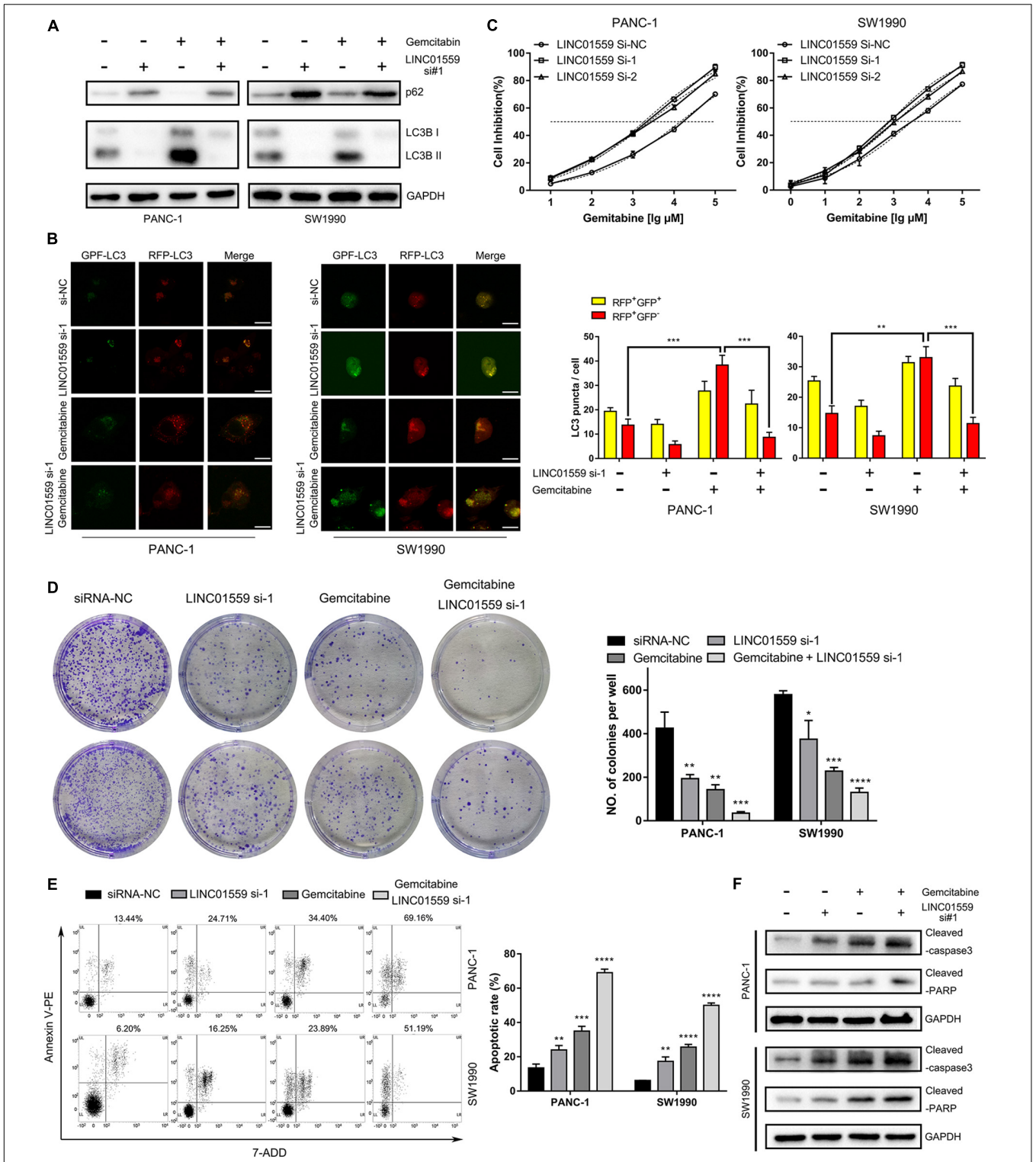


FIGURE 11 | Inhibition of LINC01559 suppressed PC cell autophagy and promoted apoptosis. **(A)** Protein expression levels of p62 and LC3B after various treatments measured by Western blot analysis. **(B)** Representative confocal images of autophagosome (yellow puncta) and autolysosome (red puncta) formation are presented in the left panel. Scale bar: 20μm. The numbers of RFP^+GFP^+ LC3 puncta and RFP^+GFP^- LC3 puncta are shown in the right panel. **(C)** The cell inhibition was calculated by the CCK-8 assay in PC cells treated with different concentrations of gemcitabine at 48 h. **(D)** Representative images from the clonogenic assays of PANC-1 and SW1990 cells with or without gemcitabine (10 μM) treatments and cultured for 14 days. **(E)** Apoptosis rate after various treatments was detected by flow cytometry. **(F)** Protein expression levels of cleaved PARP and cleaved caspase-3 after various treatments measured by Western blot analysis (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns, not significant).

may be involved in regulating the resistance and response to oxaliplatin in hepatocellular carcinoma. Then, we investigated the relationship of LINC01559 expression and chemoresistance. *In vitro* analysis showed that silencing LINC01559 suppressed the gemcitabine-induced autophagy and promoted gemcitabine-induced apoptosis, implying that inhibition of LINC01559 could be a potential therapeutic treatment for improving sensitivity to gemcitabine in PC patients.

Although there have been many reports of bioinformatic analysis of PC (Wei et al., 2019), we focused on the essential role of autophagy-related lncRNAs (ARlncRNAs) in biological characteristics of tumor malignancy and first proposed a six-ARlncRNAs signature for PC cohort. Moreover, we validated the independent prognostic value of the signature and explored in depth the clinical utility for predicting efficacy of immunotherapy and targeted therapy in PC patients. More importantly, we constructed an lncRNA-miRNA-mRNA regulatory network to better understand the potential biological mechanism. And, cMAP analysis was performed to screen potential small molecule drugs for patients with PC, which may provide clinical benefits. However, there are inevitably several limitations in our paper. First, due to the lack of valid data, our prognostic model and nomogram cannot be verified by external data. Second, the universality of the conclusion may be limited by the influences of race/ethnicity in PC patient TCGA cohorts. Moreover, despite the reports that LINC01559 regulate proliferation and migration by acting as a competing endogenous RNA of miR-1343-3p and miR-607 (Chen et al., 2020; Lou et al., 2020), the biological role of LINC01559 in regulating autophagy is obscure. Thus, we should combine multicenter clinical trials and prospective study to further prove the clinical value of the model in PC and it's essential to further elucidate the molecular mechanisms that link LINC01559 with autophagy.

In summary, our study provided a deeper understanding of the role of autophagy in biological characteristics of tumor malignancy and firstly proposed a six-ARlncRNAs signature that could provide valuable clinical utility for reliable prognostic prediction and personalized treatment of PC patients. Moreover, we identified the prognostic role of LINC01559 in PC, and targeting LINC01559 may be a potential therapeutic option for overcoming the resistance to gemcitabine in PC patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZD and XL designed the study. XL, ZD, and YS completed the experiments and analyzed the data. XL, ZD, and YL wrote the manuscript and were responsible for language revisions. WY and JW supervised the project. All authors reviewed the manuscript.

FUNDING

This study was funded by the National Natural Science Foundation of China (No. 81874062) and Youth Program of National Natural Science Foundation of China (No. 81902439).

ACKNOWLEDGMENTS

We sincerely thank the TCGA project for using their data.

REFERENCES

Bliss, L. A., Witkowski, E. R., Yang, C. J., and Tseng, J. F. (2014). Outcomes in operative management of pancreatic cancer. *J. Surg. Oncol.* 110, 592–598. doi: 10.1002/jso.23744

Castro-Oropeza, R., Melendez-Zajgla, J., Maldonado, V., and Vazquez-Santillan, K. (2018). The emerging role of lncRNAs in the regulation of cancer stem cells. *Cell. Oncol. (Dordr.)* 41, 585–603. doi: 10.1007/s13402-018-0406-4

Chan, E., Arlinghaus, L. R., Cardin, D. B., Goff, L., Berlin, J. D., Parikh, A., et al. (2016). Phase I trial of vorinostat added to chemoradiation with capecitabine in pancreatic cancer. *Radiother. Oncol.* 119, 312–318. doi: 10.1016/j.radonc.2016.04.013

Chen, B., Wang, C., Zhang, J., Zhou, Y., Hu, W., and Guo, T. (2018). New insights into long noncoding RNAs and pseudogenes in prognosis of renal cell carcinoma. *Cancer Cell Int.* 18, 157. doi: 10.1186/s12935-018-0652-6

Chen, K. D., Lin, C. C., Tsai, M. C., Huang, K. T., and Chiu, K. W. (2018). Tumor microenvironment mediated by suppression of autophagic flux drives liver malignancy. *Biomed. J.* 41, 163–168. doi: 10.1016/j.bj.2018.03.002

Chen, P., Wu, J. N., Shu, Y., Jiang, H. G., Zhao, X. H., Qian, H., et al. (2018). Gemcitabine resistance mediated by ribonucleotide reductase M2 in lung squamous cell carcinoma is reversed by GW8510 through autophagy induction. *Clin. Sci. (Lond.)* 132, 1417–1433. doi: 10.1042/CS20180010

Chen, X., Wang, J., Xie, F., Mou, T., Zhong, P., Hua, H., et al. (2020). Long noncoding RNA LINC01559 promotes pancreatic cancer progression by acting as a competing endogenous RNA of miR-13433p to upregulate RAF1 expression. *Aging* 12, 14452–14466. doi: 10.18632/aging.103487

Deist, T. M., Dankers, F., Valdes, G., Wijsman, R., Hsu, I. C., Oberije, C., et al. (2018). Machine learning algorithms for outcome prediction in (chemo)radiotherapy: an empirical comparison of classifiers. *Med. Phys.* 45, 3449–3459. doi: 10.1002/mp.12967

Donadelli, M., Costanzo, C., Beghelli, S., Scupoli, M. T., Dandrea, M., Bonora, A., et al. (2007). Synergistic inhibition of pancreatic adenocarcinoma cell growth by trichostatin A and gemcitabine. *Biochim. Biophys. Acta* 1773, 1095–1106. doi: 10.1016/j.bbamcr.2007.05.002

Donadelli, M., Costanzo, C., Faggioli, L., Scupoli, M. T., Moore, P. S., Bassi, C., et al. (2003). Trichostatin A, an inhibitor of histone deacetylases, strongly suppresses growth of pancreatic adenocarcinoma cells. *Mol. Carcinog.* 38, 59–69. doi: 10.1002/mc.10145

Dong, S., Fu, Y., Yang, K., Zhang, X., Miao, R., Long, Y., et al. (2020). linc01559 served as a potential oncogene and promoted resistance of hepatocellular

carcinoma to oxaliplatin by directly sponging miR-6783-3p. *Anticancer Agents Med. Chem.* doi: 10.2174/1871520620666200721122317 [Epub ahead of print].

Flaks, B., Moore, M. A., and Flaks, A. (1981). Ultrastructural analysis of pancreatic carcinogenesis. IV. Pseudoductular transformation of acini in the hamster pancreas during N-nitroso-bis(2-hydroxypropyl)amine carcinogenesis. *Carcinogenesis* 2, 1241–1253. doi: 10.1093/carcin/2.12.1241

Folkerts, H., Hilgendorf, S., Vellenga, E., Bremer, E., and Wiersma, V. R. (2019). The multifaceted role of autophagy in cancer and the microenvironment. *Med. Res. Rev.* 39, 517–560. doi: 10.1002/med.21531

Grixti, J. M., and Ayers, D. (2020). Long noncoding RNAs and their link to cancer. *Noncoding RNA Res.* 5, 77–82. doi: 10.1016/j.ncrna.2020.04.003

Iasonos, A., Schrag, D., Raj, G. V., and Panageas, K. S. (2008). How to build and interpret a nomogram for cancer prognosis. *J. Clin. Oncol.* 26, 1364–1370. doi: 10.1200/JCO.2007.12.9791

Iyer, M. K., Niknafs, Y. S., Malik, R., Singhal, U., Sahu, A., Hosono, Y., et al. (2015). The landscape of long noncoding RNAs in the human transcriptome. *Nat. Genet.* 47, 199–208. doi: 10.1038/ng.3192

Jiang, M. C., Ni, J. J., Cui, W. Y., Wang, B. Y., and Zhuo, W. (2019). Emerging roles of lncRNA in cancer and therapeutic opportunities. *Am. J. Cancer Res.* 9, 1354–1366.

Lamb, J., Crawford, E. D., Peck, D., Modell, J. W., Blat, I. C., Wrobel, M. J., et al. (2006). The connectivity map: using gene-expression signatures to connect small molecules, genes, and disease. *Science* 313, 1929–1935. doi: 10.1126/science.1132939

Li, Y., Li, W., Liang, B., Li, L., Wang, L., Huang, H., et al. (2016). Identification of cancer risk lncRNAs and cancer risk pathways regulated by cancer risk lncRNAs based on genome sequencing data in human cancers. *Sci. Rep.* 6:39294. doi: 10.1038/srep39294

Li, Z. N., Shu, Y., Chen, C. G., Li, X. Q., Li, M. Y., Zhao, X. H., et al. (2020). Acquired tamoxifen resistance is surmounted by GW8510 through ribonucleotide reductase M2 downregulation-mediated autophagy induction. *Biochem. Biophys. Res. Commun.* 528, 554–560. doi: 10.1016/j.bbrc.2020.05.149

Lou, C., Zhao, J., Gu, Y., Li, Q., Tang, S., Wu, Y., et al. (2020). LINC01559 accelerates pancreatic cancer cell proliferation and migration through YAP-mediated pathway. *J. Cell. Physiol.* 235, 3928–3938. doi: 10.1002/jcp.29288

Piffoux, M., Eriau, E., and Cassier, P. A. (2020). Autophagy as a therapeutic target in pancreatic cancer. *Br. J. Cancer* doi: 10.1038/s41416-020-01039-5 [Epub ahead of print].

Samstein, R. M., Lee, C. H., Shoushtari, A. N., Hellmann, M. D., Shen, R., Janjigian, Y. Y., et al. (2019). Tumor mutational load predicts survival after immunotherapy across multiple cancer types. *Nat. Genet.* 51, 202–206. doi: 10.1038/s41588-018-0312-8

Siegel, R. L., Miller, K. D., and Jemal, A. (2019). Cancer statistics, 2019. *CA Cancer J. Clin.* 69, 7–34. doi: 10.3322/caac.21551

Siegel, R. L., Miller, K. D., and Jemal, A. (2020). Cancer statistics, 2020. *CA Cancer J. Clin.* 70, 7–30. doi: 10.3322/caac.21590

Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., et al. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. U.S.A.* 102, 15545–15550. doi: 10.1073/pnas.0506580102

Tang, X. J., Wang, W., and Hann, S. S. (2019). Interactions among lncRNAs, miRNAs and mRNA in colorectal cancer. *Biochimie* 163, 58–72. doi: 10.1016/j.biochi.2019.05.010

Tang, Z., Kang, B., Li, C., Chen, T., and Zhang, Z. (2019). GEPIA2: an enhanced web server for large-scale expression profiling and interactive analysis. *Nucleic Acids Res.* 47, W556–W560. doi: 10.1093/nar/gkz430

Tian, L., Deng, Z., Xu, L., Yang, T., Yao, W., Ji, L., et al. (2018). Downregulation of ASPP2 promotes gallbladder cancer metastasis and macrophage recruitment via aPKC-iota/GLI1 pathway. *Cell Death Dis.* 9:1115. doi: 10.1038/s41419-018-1145-1

Vincent, A., Herman, J., Schulick, R., Hruban, R. H., and Goggins, M. (2011). Pancreatic cancer. *Lancet* 378, 607–620. doi: 10.1016/S0140-6736(10)62307-0

Wang, L., Bo, X., Yi, X., Xiao, X., Zheng, Q., Ma, L., et al. (2020). Exosome-transferred LINC01559 promotes the progression of gastric cancer via PI3K/AKT signaling pathway. *Cell Death Dis.* 11:723. doi: 10.1038/s41419-020-02810-5

Wei, C., Liang, Q., Li, X., Li, H., Liu, Y., Huang, X., et al. (2019). Bioinformatics profiling utilized a nine immune-related long noncoding RNA signature as a prognostic target for pancreatic cancer. *J. Cell. Biochem.* 120, 14916–14927. doi: 10.1002/jcb.28754

Yang, L., Wang, H., Shen, Q., Feng, L., and Jin, H. (2017). Long non-coding RNAs involved in autophagy regulation. *Cell Death Dis.* 8:e3073. doi: 10.1038/cddis.2017.464

YiRen, H., YingCong, Y., Sunwu, Y., Keqin, L., Xiaochun, T., Senrui, C., et al. (2017). Long noncoding RNA MALAT1 regulates autophagy associated chemoresistance via miR-23b-3p sequestration in gastric cancer. *Mol. Cancer* 16:174. doi: 10.1186/s12943-017-0743-3

Yun, C. W., and Lee, S. H. (2018). The roles of autophagy in cancer. *Int. J. Mol. Sci.* 19:3466. doi: 10.3390/ijms19113466

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Deng, Li, Shi, Lu, Yao and Wang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 12 January 2021
doi: 10.3389/fcell.2020.614178

Autophagy and Exosomes Relationship in Cancer: Friends or Foes?

Marta Colletti†, Donatella Ceglie†, Angela Di Giannatale and Francesca Nazio**

Department of Pediatric Hemato-Oncology and Cell and Gene Therapy, IRCCS, Bambino Gesù Children's Hospital, Rome, Italy

Autophagy is an intracellular degradation process involved in the removal of proteins and damaged organelles by the formation of a double-membrane vesicle named autophagosome and degraded through fusion with lysosomes. An intricate relationship between autophagy and the endosomal and exosomal pathways can occur at different stages with important implications for normal physiology and human diseases. Recent researches have revealed that extracellular vesicles (EVs), such as exosomes, could have a cytoprotective role by inducing intracellular autophagy; on the other hand, autophagy plays a crucial role in the biogenesis and degradation of exosomes. Although the importance of these processes in cancer is well established, their interplay in tumor is only beginning to be documented. In some tumor contexts (1) autophagy and exosome-mediated release are coordinately activated, sharing the molecular machinery and regulatory mechanisms; (2) cancer cell-released exosomes impact on autophagy in recipient cells through mechanisms yet to be determined; (3) exosome-autophagy relationship could affect drug resistance and tumor microenvironment (TME). In this review, we survey emerging discoveries relevant to the exosomes and autophagy crosstalk in the context of cancer initiation, progression and recurrence. Consequently, we discuss clinical implications by targeting autophagy-exosomal pathway interaction and how this could lay a basis for the purpose of novel cancer therapeutics.

Keywords: autophagy, exosome, microenvironment, cancer, target therapy

OPEN ACCESS

Edited by:
Daniel Hector Grasso, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

Reviewed by:
Ravi Manjithaya, Jawaharlal Nehru Centre for Advanced Scientific Research, India
Jafar Rezaie, Urmia University of Medical Sciences, Iran

****Correspondence:***
Angela Di Giannatale
angela.digiannatale@opbg.net
Francesca Nazio
francesca.nazio@opbg.net

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: *05 October 2020*
Accepted: *16 December 2020*
Published: *12 January 2021*

Citation:
Colletti M, Ceglie D, Di Giannatale A and Nazio F (2021) Autophagy and Exosomes Relationship in Cancer: Friends or Foes? Front. Cell Dev. Biol. 8:614178. doi: 10.3389/fcell.2020.614178

AUTOPHAGY: AN OVERVIEW

Autophagy is a self-degradative process occurring in all eukaryotic cells for maintaining homeostasis and cell survival. In basal conditions, autophagy degrades cytosolic materials such as long-lived proteins and old organelles for renewal of cellular components. During stressful conditions such as starvation or hypoxia, autophagy is induced to recycle macromolecules, providing energy and nutrients. Autophagy begins with the formation of a unique smooth double-membrane phagophore that traps cytosolic materials; after closure, autophagosome vesicle is formed and upon fusion with lysosomes, the inner membrane and the cargos are degraded and subsequently recycled (Yu et al., 2018). Three morphologically and mechanistically distinct types of autophagy have been described: macroautophagy (here referred to as autophagy), microautophagy and chaperone-mediated autophagy (CMA) (Abdrakhmanov et al., 2020). They differ substantially for cargo selection and delivery mechanism into lysosomes: macroautophagy is characterized by the formation of autophagosome, while during microautophagy the cargos are sequestered by direct invagination of the vacuole membrane. CMA only occurs in mammalian cells and uses chaperones to identify cargo proteins that contain a particular

pentapeptide motif that are translocated directly across the lysosomal membrane. Although autophagy was initially thought to be a non-selective degradation mechanism, now it is clear that it allows the removal of specific cellular components such as mitochondria (mitophagy), aggregates (aggrephagy), or invading pathogens (xenophagy) (Gatica et al., 2018).

Autophagosome formation is driven by the autophagy-related (ATG) proteins that are both spatially and temporally controlled and are divided into distinct units: ULK complex, the class III phosphatidylinositol 3-kinase (PI3K) complex, the ATG2-ATG18/WIPI4 complex, ATG9, the ATG12 conjugation system and the ATG8/LC3 conjugation system (Nishimura and Tooze, 2020).

The role of autophagy has been explored in many fields (Yang and Klionsky, 2020). In cancer progression, autophagy has a dual and paradoxical role: while at early stages autophagy acts as a tumor suppressor mechanism, in advanced stages it has a fundamental role in tumor survival being active in response to cellular stress (White, 2015; Keulers et al., 2016; Cotzomi-Ortega et al., 2018; Amaravadi et al., 2019, 2020; Mulcahy Levy and Thorburn, 2020).

EXOSOMES: BIOGENESIS, RELEASE AND FUNCTIONS

According to the International Society for Extracellular Vesicles (ISEV) which provides guidelines for the classification of extracellular vesicles (EVs), exosomes are nano-sized (30–120 nm) (Théry et al., 2018). EVs secreted by all cell types that play a role in cell-cell communication through the transfer of active biomolecules such as proteins, lipids, RNAs, DNA and microRNAs (Raposo and Stoorvogel, 2013). Exosome precursors, named intraluminal vesicles (ILVs), derive from the membrane of endocytic cisternae by inward budding of microdomains. Upon ILVs accumulation, the cisternae become multivesicular bodies (MVBs) that undergo exocytic fusion with the plasma membrane followed by release of their ILVs to the extracellular space (Raposo and Stoorvogel, 2013; Cocucci and Meldolesi, 2015). Due to the mechanism of biogenesis, the exosomal membrane has the same orientation as the parental cell plasma membrane, and it is enriched in endosome-related proteins, lipids and tetraspanins. The exact mechanisms involved in exosomes packaging have not been fully elucidated but their secretion requires formation of an endosomal-sorting complex that is required for transport (ESCRT) (Scita and Di Fiore, 2010). ESCRT is comprised of four complexes (ESCRT−0, −I, −II, and −III) and associated proteins (vacuolar protein sorting-associated protein 4 (VPS4), tumor Susceptibility 101 (Tsg101) and ALIX) (Schuh and Audhya, 2014). In addition to ESCRT, which recognizes ubiquitylated proteins, other ESCRT-independent mechanisms operate to generate exosomes (Stuffers et al., 2009). These unconventional ESCRT-independent pathways seem to be driven by the presence of certain lipids, such as ceramides and lysobisphosphatidic acid (Matsuo et al., 2004; Babst, 2011). The release of exosomes into the extracellular environment requires the transport and docking of MVBs as well as their fusion with the plasma membrane (van Niel et al., 2006). These processes need several factors including molecular switches, cytoskeleton, molecular motors and the membrane fusion apparatus. It has been proposed that exosome release is a Ca^{2+}-dependent (Savina et al., 2003) and pH-dependent (Parolini et al., 2009) process. After secretion, exosomes can be transferred to recipient cells *via* clathrin-mediated endocytosis (Tian et al., 2014), lipid raft-mediated endocytosis (Svensson et al., 2013), heparin sulfate proteoglycans-dependent endocytosis (Christianson et al., 2013), or phagocytosis (Feng et al., 2010). These pathways lead to different sorting and fate of exosomal cargo and the route by which exosomes are internalized appears to be cell and context specific. Tian et al. (2014) have showed that clathrin-mediated endocytosis and macropinocytosis are involved in the up-take of PC12-derived exosomes through a receptor-mediated mechanism. Svensson et al. (2013) have demonstrated that the signaling status of recipient cells is important in determining the pathway by which exosomes are internalized: exosomes derived from glioblastoma (GBM) cells, indeed, trigger lipid raft-mediated endocytosis where ERK activation is required. Furthermore, Christianson and co-workers provide evidences that exosomes produced by GBM cells require heparan sulfate proteoglycans for internalization and this affects the functional effects of exosomes in cancer cells (Christianson et al., 2013). Feng et al. (2010) have highlighted the role of the type of recipient cell in determining how exosomes are internalized: they have showed that phagocytic cells internalize exosomes *via* phagocytosis while in non-phagocytic cells exosomes attach to the cell membrane. In target cells, molecules carried by exosomes can trigger and influence several processes both in physiological and pathological conditions. In recent years, numerous evidence highlights the involvement of exosomes in angiogenesis promotion (Skog et al., 2008; Hong et al., 2009; Ahmadi and Rezaie, 2020), suppression of immune response (Yu et al., 2007; Clayton et al., 2008), induction of invasive (Luga et al., 2012; Guo et al., 2019; Jabbari et al., 2020a) and metastatic phenotype (Peinado et al., 2012), formation of pre-metastatic niche (Costa-Silva et al., 2015; Colletti et al., 2020). Moreover, tumoral exosomes can induce drug resistance carrying miRNAs that target antiapoptotic and immune-suppressive pathways or ABC transporters, which export chemotherapeutic agents out of recipient cells (Santos and Almeida, 2020). Given their involvement in cancer progression and their presence in different biological fluids, there have been increasing efforts toward their characterization as a source of possible diagnostic and prognostic biomarkers even in pediatric oncology (Colletti et al., 2017, 2019, 2020; Galardi et al., 2019, 2020) and as a delivery tool for biomedical applications (Rezaie et al., 2018; Rahbarghazi et al., 2019; Jabbari et al., 2020b; Wu Z. et al., 2020).

CROSSTALK BETWEEN AUTOPHAGY AND EXOSOME BIOGENESIS

An intricate relationship among autophagy and the exosome biogenesis (**Figure 1A**) occurs at different stages (Buratta et al., 2020; Hassanpour et al., 2020; Salimi et al., 2020). If in some

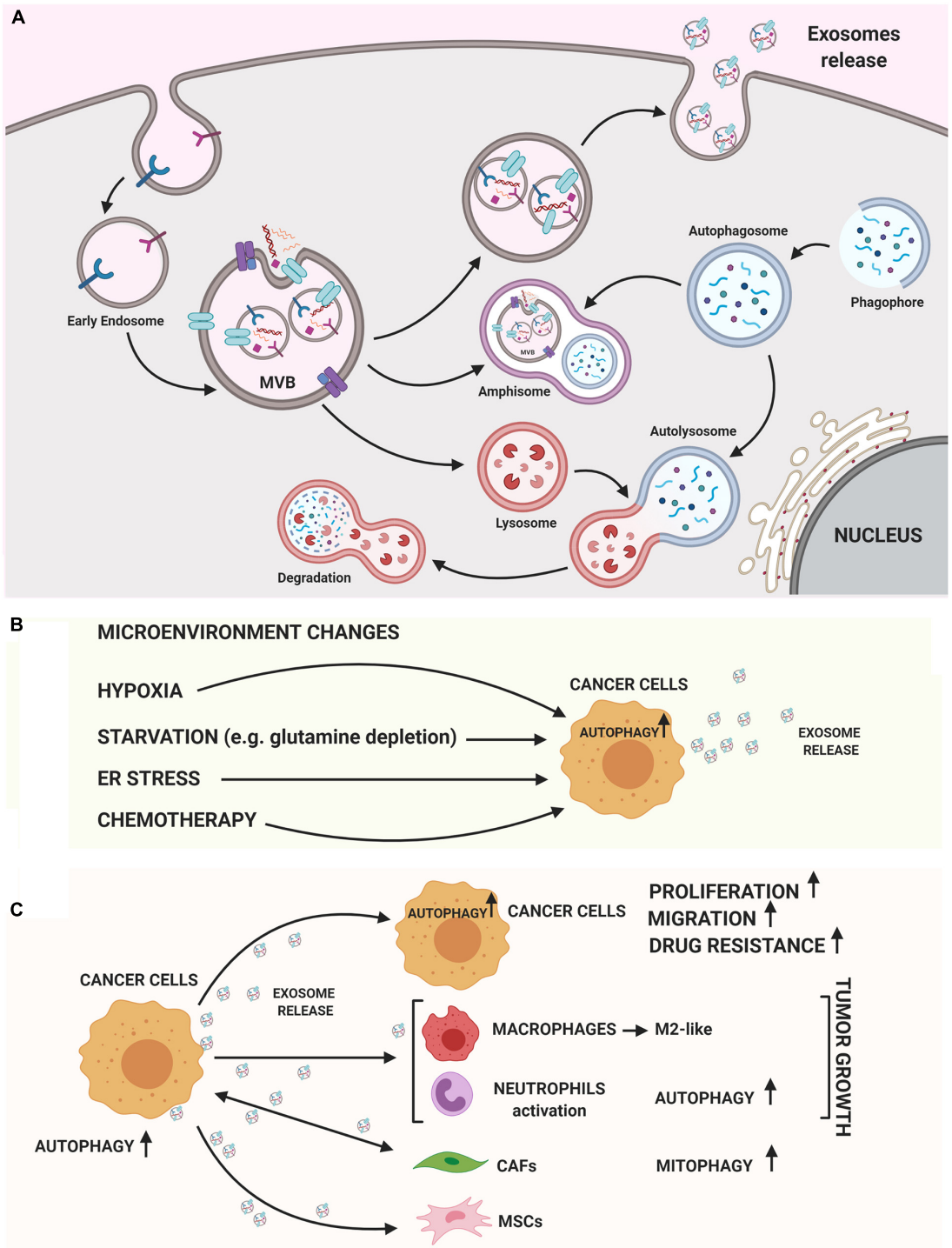


FIGURE 1 | Autophagy and exosomes relationship. **(A)** A crosstalk between exosome biogenesis and autophagy flux occurs both at molecular level and at membranous vesicles such as amphisomes. In the cytoplasm several Rab-GTPase proteins regulate the movement of vesicles between autophagy and exosomal secretory pathway. On the MVB's membrane different autophagic proteins such as LC3B, ATG5, and ATG16L1 participate to generate exosomes. Then, exosomes can carry autophagic cargo and secrete this into extracellular milieu. **(B)** Both autophagy and exosome release are strongly stimulated by TME conditions (hypoxia, starvation, ER stress) or chemotherapy treatments. **(C)** Exosomes released by cancer cells can induce autophagy in recipient cells, stimulating growth, migration and enhancing drug resistance. On the other hand, cancer cell-released exosomes can activate pro-tumoral stromal or immune cells *via* autophagy-related mechanisms or MSCs-derived exosomes may induce oncogenic autophagy in recipient cancer cells. The figure was performed with https://biorender.com.

cellular contexts autophagy and exosome production act at the same time to counter cellular stress (Kumar et al., 2014), in other circumstances the two processes can compensate each other. In fact, dysfunctional MVBs can be degraded by autophagy and the inhibition of lysosomal function or autophagy restores exosome secretion (Villarroya-Beltri et al., 2016). Moreover, EVs can have a cytoprotective role by inducing intracellular autophagy and, on the other hand, autophagy regulates the biogenesis and degradation of EVs (Xu et al., 2018). Finally, emerging evidence supports a role of both autophagy and exosomes in contributing to the export of cytokines or proteins by an unconventional secretory pathway (Ponpuak et al., 2015; Zhang et al., 2015; Kimura et al., 2017). The main advances about the crosstalk between these pathways are summarized below.

Molecular Interaction Mechanism

Some studies are emerging about how individual ATG proteins can regulate exosome biogenesis and secretion (Salimi et al., 2020). Intriguingly, it has been observed that ATG5, which participates at the stage of autophagosome precursor synthesis (Nishimura and Tooze, 2020), allows the dissociation of vacuolar proton pumps (V_1V_0-ATPase) from the MVBs, thus inhibiting the acidification of MVB-lumen and contributing to the fusion of MVB with the plasma membrane (Guo et al., 2017) in a canonical autophagy-independent manner. The treatment with V-ATP inhibitors of Atg5 knockout cells demonstrated that luminal pH plays a role in controlling whether MVBs must undergo fusion with lysosomes for degradation or with plasma membrane for exosomes release (Mauthe et al., 2018). Guo et al. (2017) have demonstrated that the down-regulation of both ATG16L1, a core autophagy protein implicated at distinct phases of autophagosome biogenesis (Nishimura and Tooze, 2020), and ATG5 reduces exosome biogenesis in breast cancer cells; this, in turn, decreases tumor metastasis. Moreover, G alpha interacting protein (GAIP) and GAIP interacting protein C-terminus (GIPC), two proteins initially identified for G-protein coupled receptor subunit GI alpha (De Vries et al., 1998), can simultaneously stimulate exosome biogenesis and autophagy flux in pancreatic tumor cells (Bhattacharya et al., 2014).

Murrow et al. (2015) have demonstrated that the inhibition of ATG12–ATG3, a complex essential for a late step of autophagosome formation (Nishimura and Tooze, 2020), changes the form of MVBs, disrupts late endosome trafficking and reduces exosome biogenesis. This occurs through an interaction between ATG12-ATG3 and ALIX, a protein implicated in membrane fission which interacts with ESCRT members involved in exosomes release. ALIX inhibition also reduces basal autophagy flux, indicating a reciprocal regulation between autophagy and exosome biogenesis. Moreover, loss of ALIX or depletion of ATG12-ATG3 complex does not impact on starvation-induced autophagy, specifying the association of different complexes which control basal and stress-induced autophagy (Murrow et al., 2015).

Interestingly, a study perfomed by Bader and collaborators reports that the transmembrane protein ATG9 is implicated in the formation of ILVs in *Drosophila melanogaster*. In basal conditions, depletion of ATG9 leads to both autophagy inhibition and decrease of the ILVs content of amphisomes and autolysosome (Bader et al., 2015).

One of the key autophagy players is MAP1LC3B, Microtubule Associated Protein 1 Light Chain 3 Beta (or LC3B). LC3B is one of the main autophagy flux markers: in the initiation step, LC3B conjugation complex induces autophagosome biogenesis through ULK activation; during the maturation step, LC3B mediates closure, fusion and transport of the autophagosome (Mizushima et al., 2011). LC3B is incorporated into autophagosome membranes but it is also recruited to single-membrane phagosomes in a process called LC3-associated phagocytosis (LAP), which does not require the formation of autophagosomes (Florey et al., 2011; Martinez et al., 2011). LC3B has been found into exosomes (LC3-I rather than LC3-II) in an ATG7-independent manner, suggesting that the LAP-like lipidation mechanism could share a non-degradative role in exosome secretion. Furthermore, a very recent work has identified a new secretory mechanism where components of LC3 conjugation complex favor the association with RNA binding proteins (RBPs) and small non-coding RNAs into EVs, resulting in their secretion outside of cells (Leidal et al., 2020). This process has been defined as LDELS: LC3-Dependent EV Loading and Secretion. Using a proximity-dependent biotinylation proteomics strategy, the authors found that this mechanism does not require canonical autophagy but only components of the LC3 conjugation machinery, linking exosome secretion pathway, extracellular RNA release and autophagy in a very fascinating way. Finally, although not designated as ATGs, soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins are also an example of the interplay between autophagy and exosome secretion (Zhao and Zhang, 2019). SNARE complexes (such as STX17-SNAP29-VAMP7/VAMP8 or STX7-SNAP29-YKT6) mediate autophagosome-lysosome fusion; however, secretory autophagy requires different SNAREs than degradative autophagy (such as Sec22b), adding further to the complexity of this crosstalk (Zhao and Zhang, 2019).

Interestingly, growing evidence indicates that exosomes could contain ATG proteins. For example, Sequestosome 1 (SQSTM1), a classical receptor of autophagy, Neighbor Of BRCA1 Gene 1 Protein (NBR1), a selective autophagy receptor, WD Repeat Domain, Phosphoinositide Interacting 2 (WIPI2), a component of the autophagy machinery, or LC3 were found into exosomal fractions in PC-3 cells; intriguingly, both SQSTM1 and CD63, used as a representative exosomal marker protein, were found in the same MVB-like organelles inside the cells (Hessvik et al., 2016). Minakaki et al. (2018) have discovered that, in neuronal cells, autophagy inhibition increases alpha-synuclein levels in EVs released in human cerebrospinal fluid. These vesicles are biochemically characterized by the presence of both LC3-II and SQSTM1 together with classical MVB-EV markers. This study provides, for the first time, the presence of EVs with a hybrid autophagosome-exosome-like profile.

Vesicular Interaction Mechanism

Antagonist interaction between autophagy and exosomes release has been well-defined in the context of amphisomes biogenesis. Amphisomes are degradative hybrid compartments formed after

fusion between autophagosomes and MVBs, which can then fuse with lysosomes (Liou et al., 1997). As an example, autophagy induction supports MVB-autophagosome fusion and leads to a reduction of exosomes release (Fader et al., 2008). On the other hand, autophagy inhibition rescues exosome secretion, suggesting an involvement of autophagy in the lysosome-dependent degradation of MVBs (Villarroya-Beltri et al., 2016).

Recently, using biochemical, electron microscopy and fluorescence microscopy-based approaches, Ariotti et al. (2020) dissect a novel autophagy-based secretion of Caveolin 1 (CAV1) in addition to conventional exosome-based release. In details, in pancreatic cancer cells, they identified a novel class of exosomes, enriched in CAV1 (50–60 copies), and released *via* a non-canonical secretory autophagy pathway.

AUTOPHAGY AND EXOSOMES RELATIONSHIP IN CANCER

Although the importance of autophagy and exosomes in tumor progression is well-documented (Yu et al., 2007; Clayton et al., 2008; Skog et al., 2008; Hong et al., 2009; Svensson et al., 2013; Keulers et al., 2016; Ahmadi and Rezaie, 2020; Amaravadi et al., 2020), in recent years the understanding of their connection and interplay in cancer has aroused a lot of interest (**Table 1**; Kulshreshtha et al., 2007; Bellot et al., 2009; Chiavarina et al., 2010; Mazure and Pouysségur, 2010; White et al., 2010; Aga et al., 2014; Dutta et al., 2014; Li et al., 2016; Zhang et al., 2018, 2019; Jin et al., 2019; Meng et al., 2019; Shao et al., 2019; Wang et al., 2019, 2020; Yeon et al., 2019; Yuwen et al., 2019; Zeng et al., 2019; Zhou et al., 2019; Dai et al., 2020; Han et al., 2020; Huang et al., 2020; Kulkarni et al., 2020; Kumar and Deep, 2020; Liu et al., 2020; Sung et al., 2020; Wu X. et al., 2020; Yao et al., 2020).

Are Autophagy and Exosome-Mediated Release Coordinately Induced During Carcinogenesis?

In tumor cells, both autophagy and exosome release are strongly activated, suggesting that both these pathways are a part of cancer cells response (**Figure 1B**). This coordinated activation may represent an adaptive stress response, although the molecular details are not yet understood. Hypoxic tumor microenvironment (TME) is a key feature in many solid tumors and it is associated with unfavorable prognosis. In tumors, both starvation and hypoxia induce autophagy, which avoids inflammation and cell death (Bellot et al., 2009; Chiavarina et al., 2010; Mazure and Pouysségur, 2010; White et al., 2010). Many studies have shown that cancer cells secrete a higher number of exosomes under hypoxic conditions (Kumar and Deep, 2020) and hypoxia is able to alter the proteomic and nuclear acid profiles of exosomes (Meng et al., 2019). Interestingly, HIF-1α was found in exosomes with transcriptional activity (Aga et al., 2014), representing a potential cancer biomarker. In addition, several miRNAs under the transcriptional control of HIF-1α are enriched in EV derived from hypoxic cells; among these, miR-23a targets BCL2 Interacting Protein 3 Like (BNIP3L), a crucial mitophagy receptor (Kulshreshtha et al., 2007). Moreover, high levels of the HIF1-α transcriptional target *BCL2 Interacting Protein 3 (BNIP3)* mRNA, another mitophagy receptor, are found in EV produced by hypoxic glioma cells (Kucharzewska et al., 2013).

In cancer cells autophagy and exosome release may be concomitantly up-regulated in response to other cellular stressors such as unfolding protein response (UPR) and endoplasmic reticulum (ER) stress. ER stress is known to increase autophagy in several types of normal and tumor cells (Verfaillie et al., 2010; Corazzari et al., 2017). Kanemoto et al. (2016) found that MVB formation and exosomes release are enhanced by ER stress; furthermore, the down-regulation of both Inositol-Requiring Protein 1 (IRE1α) and PKR-like ER kinase (PERK), two key players of UPR pathway, impacts on exosome production. In addition, the spliced form of X-box binding protein 1 (sXBP1), a key transcription factor that promotes UPR, was found in exosomes, suggesting the transmission outside the cell of UPR mechanism, following exposure to stresses (Hosoi et al., 2018).

Very recently, using *Drosophila* model and human cell lines, Fan et al. (2020) have found that glutamine depletion induces secretion of exosomes carrying exclusive cargos created in Rab11−positive recycling endosomal MVBs. Interestingly, the release of exosomes from glutamine depleted HCT116 cells stimulate angiogenesis and enhances tumor cell proliferation. Glutamine depletion is likely to be an autophagy inducer determining tumor growth (Tan et al., 2017), supporting the concept of a strict connection between autophagy and exosome secretion as a part of neoplastic cells response.

Beside microenvironmental conditions, up-regulation of both autophagy and exosome release has been well-recognized after chemotherapy treatments (Bandari et al., 2018; Yun and Lee, 2018; Ab Razak et al., 2019; Kang et al., 2020) and in mediating chemoresistance (Yun and Lee, 2018; Ender et al., 2019; Nazio et al., 2019; Steinbichler et al., 2019). For example, exosomes can carry functional plasma membrane transporter proteins from resistant cells to their drug-sensitive ones (Lu et al., 2013) or can sequestrate drugs reducing their concentration (Goler-Baron et al., 2012). It is unknown whether up-regulation of both autophagy and exosome secretion is part of the resistance mechanism or a consequence of cellular phenotype changes. Nevertheless, autophagy inhibiiton and modulation of exosome release may serve for therapeutic approaches and needs to be investigated.

How Do Exosomes Released by Tumor Influence Autophagy in Recipient Cells?

Specific cancer exosomal miRNAs and proteins seem to have a crucial role in determining an ATG response (**Figure 1C**; Jin et al., 2019; Shao et al., 2019; Yuwen et al., 2019; Han et al., 2020; Kulkarni et al., 2020; Wang et al., 2020). Recently, exosomes carrying miR-1910-3p secreted by breast cancer cells have been found to promote tumor development inducing proliferation, migration and autophagy in recipient mammary epithelial cells and breast cancer cells (**Table 1**; Wang et al., 2020). Several recent studies have shown that specific exosomal miRNAs

TABLE 1 | Summary of the exosomal molecules that regulate autophagy in target cells or whose release is regulated by autophagy in cancer models.

Exosomal molecule	Target molecule/pathway	Cell releasing exosomes	Target cells	Autophagy in cells releasing exosomes	Autophagy in target cells	Effect	References
miR-1910-3p	MTMR3	Breast cancer	Cancer cells	n.d.	Induction	Increased proliferation and migration	Wang et al., 2020
miR-1229-5p, miR-1246, miR-21-5p, miR-96-5p	Autophagy pathway	Serum of CRC patients	n.d.	n.d.	n.d.	Chemoresistance	Jin et al., 2019
miR-454-3p	ATG12	Serum of glioma patients; glioma cells	Glioma cells	n.d.	Suppression	Tumor suppression	Shao et al., 2019
miR-567	ATG5	Breast cancer	Cancer cells	n.d.	Suppression	Increased trastuzumab sensitivity	Han et al., 2020
miR-425-3p	AKT1	NSCLC	Cancer cells	n.d.	Induction	Increased platinum chemoresistance	Yuwen et al., 2019
miR-30a	BECLIN1/Bcl2	OSCC	Cancer cells	n.d	Induction	Decreased cisplatin sensitivity	Kulkarni et al., 2020
miR-19a-3p	phosphatase and tensin homolog/AKT/mTOR signaling pathway	SHSY5Y (NB cells)	Microglia cells	n.d.	Suppression	Dysfunction of autophagy in recipient cells	Zhou et al., 2019
CircNRIP1	AKT1/mTOR	Gastric cancer	Cancer cells	n.d.	Suppression	Increased tumor progression	Zhang et al., 2019
Circ-PVT1	miR-30a/YAP1	Gastric cancer	Cancer cells	n.d	Induction	Increased cisplatin chemoresistance	Yao et al., 2020
WNT1	WISP-3	$CT26^{Flag-CAGE1}$ (mouse colon cancer cells)	CT26, mast cells and macrophages	Increased	Induction	Increased tumorigenic potential	Yeon et al., 2019
HMGB1	TLR4/NF-kB pathway	Gastric cancer	Neutrophils	n.d.	Induction	Increased pro-tumor activation of neutrophils	Zhang et al., 2018
n.d.	n.d.	Breast cancer	Mammary epithelial cells	n.d.	Induction	ROS production, DNA damage response, release of tumor promoting factors	Dutta et al., 2014
n.d.	ATG5	BMMSC	OS cells	n.d.	Induction	Promotion of proliferation, migration and invasion	Huang et al., 2020
LMP1	p65/NF-kB pathway	NPC cells	NFs, CAFs	n.d.	Induction	Promotion of proliferation, migration and radiation resistance of NPC cells	Wu X. et al., 2020
MALAT1	AKT1/mTOR	Lung carcinoma cells	DCs	n.d.	Induction	T cells proliferation inhibition	Liu et al., 2020
KRASG12D	STAT3 pathway	PDAC	Macrophages	Increased	n.d.	Polarization of macrophages into M2-like TAM	Dai et al., 2020
ITGB4	BNIP3L	Breast cancer	CAFs	n.d	Induction (Mitophagy)	Induced tumor progression	Sung et al., 2020
LC3, SQSTM1, SQSTM1–349, NBR1, NDP52	n.d.	Breast cancer cells	Breast cancer cells	Increased	Induction	Increased proliferation	Wang et al., 2019
VEGF,miR-9	n.d.	HUVEC	HCC cell lines	Decreased	Induction	Increased angiogenesis	Zeng et al., 2019
n.d.	n.d.	Adenocarcinoma cells	Adenocarcinoma cells	n.d.	Induction	Chemoresistance	Li et al., 2016

MTMR3, myotubularin related protein 3; CRC, colorectal cancer; NSCLC, non-small cell lung cancer; OSCC, oral squamous cell carcinoma; NB, neuroblastoma; YAP-1, yes associated protein-1; WISP-3, WNT1-inducible-signaling pathway protein 3; HMGB1, high mobility group box 1; TLR4, toll like receptor 4; BMSC, bone marrow mesenchymal stem cells; OS, osteosarcoma; LMP1, latent membrane protein 1; NPC, nasopharyngeal carcinoma; NFs, normal fibroblasts; CAFs, cancer associated fibroblasts; MALAT1, metastasis associated lung adenocarcinoma transcript 1; DCs, dendrytic cells; PDAC, pancreatic ductal adenocarcinoma; ITGB4, integrin beta 4; BNIP3L, BCL2 interacting protein 3 like; SQSTM1, sequestrome1; VEGF, vascular endothelial growth factor; HUVEC, human umbilical vein endothelial cells; HCC, hepatocellular carcinoma; n.d., not determined.

regulate autophagy-dependent therapy resistance in recipient cells (Kaminskyy et al., 2012; Zhou et al., 2012; Dutta et al., 2014; Zhang et al., 2018; Deng et al., 2019; Huang et al., 2020; Kang et al., 2020). In breast cancer, exosomal miR-567 down-regulates ATG5 and consequently autophagy, reversing trastuzumab resistance (Dutta et al., 2014). In cisplatin-resistant non-small cell lung cancer (NSCLC), exosomal miR-425-3p down-regulates AKT1 inducing autophagy and leading to therapeutic failure both in early and advanced stages (Yuwen et al., 2019). Also, in oral squamous cell carcinoma (OSCC), exosomal miR-30a modulates cisplatin-sensitivity reducing autophagy *via* Beclin1 and Bcl2 (Kulkarni et al., 2020). These studies are in support for the use of exosome- mediated miRNA delivery as an effective therapeutic approach. In a very recent paper, exosomal circRNA-plasmacytoma variant translocation 1 (circ-PVT1) intensifies cisplatin-resistant gastric cells through modulating autophagy, invasion, and apoptosis; circ-PVT1 negatively controls miR-30a-5p that, in turn, regulates Yes-associated protein 1 (YAP1) levels (Yao et al., 2020). Besides general autophagy, there is some evidence that also specific forms of autophagy could be modulated by exosomes. Liu et al. (2019) showed that hepatitis B virus (HBV)-infected liver cancer cells-derived exosomes promote liver cancer chemoresistance by modulating the CMA pathway. Specifically, an increased expression of lysosome associated-membrane protein type 2A (LAMP2A), a membrane protein that acts as a specific receptor for the CMA, was observed in cells treated with HBV-associated exosomes and this is associated with a down-regulation of cell death after oxaliplatin treatment due to the activation of the CMA pathway. This is the first study investigating the connection between CMA and exosome release in cancer drug resistance; it proposes the targeting of exosomes to increase chemosensitivity in patients with HBV-liver cancer.

In addition to having a role on tumor cells communication, cancer cell-released exosomes are also able to modulate ATG mechanisms in surrounding stromal and immune cells to support tumor progression. In a study performed by Zhang et al. (2018) suggest that gastric cancer cell-derived exosomes induce autophagy and pro-tumor activation of neutrophils, which, in turn, promote gastric cancer cell migration. Other authors have begun to investigate the importance of exosomes-autophagy interplay between normal and neoplastic cells in supporting carcinogenesis. For example, mesenchymal stem cells (MSCs)-derived exosomes have been identified to modulate autophagy in pathological conditions such as during ischemia or spinal cord injury (Baixauli et al., 2014; Tian et al., 2019) and, more recently, in cancer (Huang et al., 2020). Huang et al. (2020) indeed, found that MSCs-derived exosomes promote osteosarcoma development and invasion by inducing autophagy.

Dai et al. (2020) found that extracellular $KRAS^{G12D}$ is packaged into exosomes and transferred, through them, from cancer cells to macrophages. In pancreatic ductal adenocarcinoma (PDAC), G12D is the most frequent mutation in KRAS. In this work the authors demonstrate that oxidative-stress induced autophagy regulates $KRAS^{G12D}$ protein release from PDAC cells, and this drives macrophages polarization into pro-tumor M2-like tumor-associated macrophages. Given that autophagy can influence exosome release, a novel study discovers a potential strategy to counteract esophangeal squamous cell carcinoma (ESCC) growth by affecting autophagy and exosome-mediated paracrine senescence (Zheng et al., 2020). Sulforaphane, an isothiocyanate derived from cruciferous vegetables, inhibited fusion process between autophagosome and lysosome resulting in significantly higher exosome release; these exosomes evidently trigger senescence of receipt ESCC cells in a ROS-mTOR-dependent manner. This is in line with the idea that defects in autophagy avoid the effective degradation of intracellular aggregates and exosome discharge may be increased to improve the proteotoxic stress. Another study proposes a link between mitochondria-selective autophagy and exosome content in cancer. Sung et al. (2020) reported that triple negative breast cancer-derived exosomal Integrin beta 4 (ITGB4) induces a metabolic reprogramming in cancer-associated fibroblasts (CAFs) that, in turn, supports tumor progression. Exosomal ITGB4 triggers the conversion of pyruvate to lactate in CAFs *via* BCL2 Interacting Protein 3 Like (BNIP3L)-dependent mitophagy. The produced lactate is released in the extracellular space and then taken-up by breast cells. This study suggests that ITGB4-induced mitophagy could be a novel target for cancer therapy.

Unveiling Exosomal Contents as New Frontier for Autophagy Modulation and Cancer Treatment

In the era of precision medicine, the development of targeted drugs is also addressing several efforts in investigating new pharmaceutical compound that can modulate autophagy, overcoming the stress tolerance of the tumor and undermining the mechanism of survival of tumor cells. In cancer biology, autophagy plays dual role in both tumor promotion and suppression. In this context the choice to induce or inhibit autophagy is related to the role of autophagy in each specific cancer. A large number of clinical trials using autophagy inhibitors (Malhotra et al., 2019) or activators (Geissler et al., 2016; Rodríguez-Perálvarez et al., 2018; Kulka et al., 2020) are ongoing and, when used in association with anti-cancer drugs, can sensitize chemoresistant cells to treatment (Singh et al., 2018). Chloroquine/Hydroxychloroquine is the only autophagy inhibitor that has been approved by the FDA; however, it also has many off-target effects and the majority of clinical trials have been performed in patients with no specific selected criteria beyond the tumor type.

Given the close relationship between autophagy and exosome pathways in cancer, a better understanding of the biological basis of this complex dialog will help to design specific therapeutic strategy (Lin et al., 2019), such as nano-carriers therapy, to modulate autophagy. Although the use of nanotechnology for the delivery of drugs/biological products targeting autophagy is largely unexplored, investigation of exosome cargo contents could offer opportunities for affecting autophagy in a specific tumor context. In particular, for the treatment of personalized cancer, antagonistic oligonucleotides (antagomiRs, anti-miRs) may be designed for the development of autophagy-modulating

therapy, increasing cell chemo-sensitivity and overcoming drug resistance. Proof-of-concept studies are required to understand the role of autophagy in each tumoral context and whether triggering or suppressing autophagy (by specific miRNAs/anti-MiRs) could counteract tumor aggressiveness and progression. To this regard, nanoparticles as miRNAs/miRs delivery systems for modulation of autophagy could be a promising therapeutic strategy.

CONCLUSION

Autophagy and exosome pathways are strictly interconnected at several levels. In cancer, increasing evidence discussed above indicate a crucial interplay between these processes. Although exosomes control of autophagy is context-dependent, targeting the exosomal pathway to modulate autophagy may suggest a basis for aiming novel cancer therapeutics that need to be further studied. Moreover, the biomarker application of the regulatory factors of both autophagy and exosome signaling has been proposed. However, the effects of their interaction are intricate and TME-dependent and therefore need further valuations.

AUTHOR CONTRIBUTIONS

FN conceptualized the work and together with AD, DC, and MC prepared a draft of the manuscript text. MC contributed to the figures development. FN, AD, and MC critically reviewed and edited the manuscript. All authors discussed the reported information and commented on the manuscript.

FUNDING

This work was supported by grants from the Ministero della Salute (GR-2016-02364088 to AD) and Ricerca Corrente (to AD and FN). Dr. Marta Colletti (MC) was supported by Fondazione Umberto Veronesi.

ACKNOWLEDGMENTS

We thank Dr. Valentina Polcini for proofreading of the manuscript.

REFERENCES

Ab Razak, N. S., Ab Mutalib, N. S., Mohtar, M. A., and Abu, N. (2019). Impact of chemotherapy on extracellular vesicles: understanding the chemo-EVs. *Front. Oncol.* 9:1113. doi: 10.3389/fonc.2019.01113

Abdrakhmanov, A., Gogvadze, V., and Zhivotovsky, B. (2020). To eat or to die: deciphering selective forms of autophagy. *Trends Biochem. Sci.* 45, 347–364. doi: 10.1016/j.tibs.2019.11.006

Aga, M., Bentz, G. L., Raffa, S., Torrisi, M. R., Kondo, S., Wakisaka, N., et al. (2014). Exosomal HIF1α supports invasive potential of nasopharyngeal carcinoma-associated LMP1-positive exosomes. *Oncogene* 33, 4613–4622. doi: 10.1038/onc.2014.66

Ahmadi, M., and Rezaie, J. (2020). Tumor cells derived-exosomes as angiogenenic agents: possible therapeutic implications. *J. Transl. Med.* 18:249. doi: 10.1186/s12967-020-02426-5

Amaravadi, R. K., Kimmelman, A. C., and Debnath, J. (2019). Targeting autophagy in cancer: recent advances and future directions. *Cancer Discov.* 9, 1167–1181. doi: 10.1158/2159-8290

Amaravadi, R. K., Kimmelmann, A. C., and White, E. (2020). Recent insights into the function of autophagy in cancer. *Genes Dev.* 30, 1913–1930. doi: 10.1101/gad.287524.116

Ariotti, N., Wu, Y., Okano, S., Gambin, Y., Follett, J., Rae, J., et al. (2020). An inverted CAV1 (caveolin 1) topology defines novel autophagy-dependent exosome secretion from prostate cancer cells. *Autophagy*. doi: 10.1080/15548627.2020.1820787 [Epub ahead of print].

Babst, M. (2011). MVB vesicle formation: ESCRT-dependent. ESCRT-independent and everything in between. *Curr. Opin. Cell Biol.* 23, 452–457. doi: 10.1016/j.ceb.2011.04.008

Bader, C. A., Shandala, T., Ng, Y. S., Johnson, I. R. D., and Brooks, D. A. (2015). Atg9 is required for intraluminal vesicles in amphisomes and autolysosomes. *Biol. Open* 4, 1345–1355. doi: 10.1242/bio.013979

Baixauli, F., López-Otín, C., and Mittelbrunn, M. (2014). Exosomes and autophagy: coordinated mechanisms for the maintenance of cellular fitness. *Front. Immunol.* 5:403. doi: 10.3389/fimmu.2014.00403

Bandari, S. K., Purushothaman, A., Ramani, V. C., Brinkley, G. J., Chandrashekar, D. S., Varambally, S., et al. (2018). Chemotherapy induces secretion of exosomes loaded with heparanase that degrades extracellular matrix and impacts tumor and host cell behavior. *Matrix Biol.* 65, 104–118. doi: 10.1016/j.matbio.2017.09.001

Bellot, G., Garcia-Medina, R., Gounon, P., Chiche, J., Roux, D., Pouysségur, J., et al. (2009). Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. *Mol. Cell. Biol.* 29, 2570–2581. doi: 10.1128/MCB.00166-09

Bhattacharya, S., Pal, K., Sharma, A. K., Dutta, S. K., Lau, J. S., Yan, I. K., et al. (2014). GAIP interacting protein C-terminus regulates autophagy and exosome biogenesis of pancreatic cancer through metabolic pathways. *PLoS One* 9:e114409. doi: 10.1371/journal.pone.0114409

Buratta, S., Tancini, B., Sagini, K., Delo, F., Chiaradia, E., Urbanelli, L., et al. (2020). Lysosomal exocytosis, exosome release and secretory autophagy: the autophagic- and endo-lysosomal systems go extracellular. *Int. J. Mol. Sci.* 21:2576. doi: 10.3390/ijms21072576

Chiavarina, B., Whitaker-Menezes, D., Migneco, G., Martinez-Outschoorn, U. E., Pavlides, S., Howell, A., et al. (2010). HIF1-alpha functions as a tumor promoter in cancer associated fibroblasts, and as a tumor suppressor in breast cancer cells: Autophagy drives compartment-specific oncogenesis. *Cell Cycle* 9, 3534–3551. doi: 10.4161/cc.9.17.12908

Christianson, H. C., Svensson, K. J., van Kuppevelt, T. H., Li, J. P., and Belting, M. (2013). Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity. *Proc. Natl. Acad. Sci. U.S.A.* 110, 17380–17385. doi: 10.1073/pnas.1304266110

Clayton, A., Mitchell, J. P., Court, J., Linnane, S., Mason, M. D., and Tabi, Z. (2008). Human tumor-derived exosomes down-modulate NKG2D expression. *J. Immunol.* 180, 7249–7258. doi: 10.4049/jimmunol.180.11.7249

Cocucci, E., and Meldolesi, J. (2015). Ectosomes and exosomes: shedding the confusion between extracellular vesicles. *Trends Cell Biol.* 25, 364–372. doi: 10.1016/j.tcb.2015.01.004

Colletti, M., Paolini, A., Galardi, A., Di Paolo, V., Pascucci, L., Russo, I., et al. (2019). Expression profiles of exosomal miRNAs isolated from plasma of patients with desmoplastic small round cell tumor. *Epigenomics* 11, 489–500. doi: 10.2217/epi-2018-0179

Colletti, M., Petretto, A., Galardi, A., Di Paolo, V., Tomao, L., Lavarello, C., et al. (2017). Proteomic analysis of neuroblastoma-derived exosomes: new insights into a metastatic signature. *Proteomics* 17:1600430. doi: 10.1002/pmic.201600430

Colletti, M., Tomao, L., Galardi, A., Paolini, A., Di Paolo, V., De Stefanis, C., et al. (2020). Neuroblastoma-secreted exosomes carrying miR-375 promote osteogenic differentiation of bone-marrow mesenchymal stromal cells. *J. Extracell. Vesicles* 9:1774144. doi: 10.1080/20013078.2020.1774144

Corazzari, M., Gagliardi, M., Fimia, G. M., and Piacentini, M. (2017). Endoplasmic reticulum stress, unfolded protein response, and cancer cell fate. *Front. Oncol.* 7:78. doi: 10.3389/fonc.2017.00078

Costa-Silva, B., Aiello, N. M., Ocean, A. J., Singh, S., Zhang, H., Thakur, B. K., et al. (2015). Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nat. Cell Biol.* 17, 816–826. doi: 10.1038/ncb3169

Cotzomi-Ortega, I., Aguilar-Alonso, P., Reyes-Leyva, J., and Maycotte, P. (2018). Autophagy and its role in protein secretion: implications for cancer therapy. *Mediators Inflamm.* 2018:4231591. doi: 10.1155/2018/4231591

Dai, E., Han, L., Liu, J., Xie, Y., Kroemer, G., Klionsky, D. J., et al. (2020). Autophagy-dependent ferroptosis drives tumor-associated macrophage polarization via release and uptake of oncogenic KRAS protein. *Autophagy* 16, 2069–2083. doi: 10.1080/15548627.2020.1714209

De Vries, L., Lou, X., Zhao, G., Zheng, B., and Farquhar, M. G. (1998). GIPC, a PDZ domain containing protein, interacts specifically with the C terminus of RGS-GAIP. *Proc. Natl. Acad. Sci. U.S.A.* 95, 12340–12345. doi: 10.1073/pnas.95.21.12340

Deng, S., Shanmugam, M. K., Kumar, A. P., Yap, C. T., Sethi, G., and Bishayee, A. (2019). Targeting autophagy using natural compounds for cancer prevention and therapy. *Cancer* 125, 1228–1246. doi: 10.1002/cncr.31978

Dutta, S., Warshall, C., Bandyopadhyay, C., Dutta, D., and Chandran, B. (2014). Interactions between exosomes from breast cancer cells and primary mammary epithelial cells leads to generation of reactive oxygen species which induce DNA damage response, stabilization of p53 and autophagy in epithelial cells. *PLoS One* 9:e97580. doi: 10.1371/journal.pone.0097580

Ender, F., Von Bubnoff, N., and Gieseler, F. (2019). Extracellular vesicles: subcellular organelles with the potential to spread cancer resistance. *Anticancer Res.* 39, 3395–3404. doi: 10.21873/anticanres.13483

Fader, C. M., Sánchez, D., Furlán, M., and Colombo, M. I. (2008). Induction of autophagy promotes fusion of multivesicular bodies with autophagic vacuoles in k562 cells. *Traffic* 9, 230–250. doi: 10.1111/j.1600-0854.2007.00677.x

Fan, S. J., Kroeger, B., Marie, P. P., Bridges, E. M., Mason, J. D., McCormick, K., et al. (2020). Glutamine deprivation alters the origin and function of cancer cell exosomes. *EMBO J.* 39:e103009. doi: 10.15252/embj.2019103009

Feng, D., Zhao, W. L., Ye, Y. Y., Bai, X. C., Liu, R. Q., Chang, L. F., et al. (2010). Cellular internalization of exosomes occurs through phagocytosis. *Traffic* 11, 675–687. doi: 10.1111/j.1600-0854.2010.01041.x

Florey, O., Kim, S. E., Sandoval, C. P., Haynes, C. M., and Overholtzer, M. (2011). Autophagy machinery mediates macroendocytic processing and entotic cell death by targeting single membranes. *Nat. Cell Biol.* 13, 1335–1343. doi: 10.1038/ncb2363

Galardi, A., Colletti, M., Di Paolo, V., Vitullo, P., Antonetti, L., Russo, I., et al. (2019). Exosomal MiRNAs in pediatric cancers. *Int. J. Mol. Sci.* 20:4600. doi: 10.3390/ijms20184600

Galardi, A., Colletti, M., Lavarello, C., Di Paolo, V., Mascio, P., Russo, I., et al. (2020). Proteomic profiling of retinoblastoma-derived exosomes reveals potential biomarkers of vitreous seeding. *Cancers* 12:1555. doi: 10.3390/cancers12061555

Gatica, D., Lahiri, V., and Klionsky, D. J. (2018). Cargo recognition and degradation by selective autophagy. *Nat. Cell Biol.* 20, 233–242. doi: 10.1038/s41556-018-0037-z

Geissler, E. K., Schnitzbauer, A. A., Zülke, C., Lamby, P. E., Proneth, A., Duvoux, C., et al. (2016). Sirolimus use in liver transplant recipients with hepatocellular carcinoma: a randomized, multicenter, open-label phase 3 trial. *Transplantation* 100, 116–125. doi: 10.1097/TP.0000000000000965

Goler-Baron, V., Sladkevich, I., and Assaraf, Y. G. (2012). Inhibition of the PI3K-Akt signaling pathway disrupts ABCG2-rich extracellular vesicles and overcomes multidrug resistance in breast cancer cells. *Biochem. Pharmacol.* 83, 1340–1348. doi: 10.1016/j.bcp.2012.01.033

Guo, D., Lui, G. Y. L., Lai, S. L., Wilmott, J. S., Tikoo, S., Jackett, L. A., et al. (2019). RAB27A promotes melanoma cell invasion and metastasis via regulation of pro-invasive exosomes. *Int. J. Cancer* 144, 3070–3085. doi: 10.1002/ijc.32064

Guo, H., Chitiprolu, M., Roncevic, L., Javalet, C., Hemming, F. J., Trung, M. T., et al. (2017). Atg5 disassociates the V1V0-ATPase to promote exosome production and tumor metastasis independent of canonical macroautophagy. *Dev. Cell* 43, 716–730.e7. doi: 10.1016/j.devcel.2017.11.018 716-730.e7,

Han, M., Hu, J., Lu, P., Cao, H., Yu, C., Li, X., et al. (2020). Exosome-transmitted miR-567 reverses trastuzumab resistance by inhibiting ATG5 in breast cancer. *Cell Death Dis.* 11:43. doi: 10.1038/s41419-020-2250-5

Hassanpour, M., Rezabakhsh, A., Rezaie, J., Nouri, M., and Rahbarghazi, R. (2020). Exosomal cargos modulate autophagy in recipient cells via different signaling pathways. *Cell Biosci.* 10:92. doi: 10.1186/s13578-020-00455-7

Hessvik, N. P., Øverbye, A., Brech, A., Lyngaas Torgersen, M., Jakobsen, I. S., Alicia, L., et al. (2016). PIKfyve inhibition increases exosome release and induces secretory autophagy. *Cell. Mol. Life Sci.* 73, 4717–4737. doi: 10.1007/s00018-016-2309-8

Hong, B. S., Cho, J. H., Kim, H., Choi, E. J., Rho, S., Kim, J., et al. (2009). Colorectal cancer cell-derived microvesicles are enriched in cell cycle-related mRNAs that promote proliferation of endothelial cells. *BMC Genomics* 10:556. doi: 10.1186/1471-2164-10-556

Hosoi, T., Nakashima, M., and Ozawa, K. (2018). Incorporation of the endoplasmic reticulum stress-induced spliced form of XBP1 mRNA in the exosomes. *Front. Physiol.* 9:1357. doi: 10.3389/fphys.2018.01357

Huang, Y., Liu, W., He, B., Lei, W., Shu, H., Sun, L., et al. (2020). Exosomes derived from bone marrow mesenchymal stem cells promote osteosarcoma development by activating oncogenic autophagy. *J. Bone Oncol.* 21:100280. doi: 10.1016/j.jbo.2020.100280

Jabbari, N., Akbariazar, E., Feqhhi, M., Rahbarghazi, R., and Rezaie, J. (2020a). Breast cancer-derived exosomes: Tumor progression and therapeutic agents. *J. Cell. Physiol.* 235, 6345–6356. doi: 10.1002/jcp.29668

Jabbari, N., Karimipour, M., Khaksar, M., Akbariazar, E., Heidarzadeh, M., Mojarad, B., et al. (2020b). Tumor-derived extracellular vesicles: insights into bystander effects of exosomes after irradiation. *Lasers Med. Sci.* 35, 531–545. doi: 10.1007/s10103-019-02880-8

Jin, G., Liu, Y., Zhang, J., Bian, Z., Yao, S., Fei, B., et al. (2019). A panel of serum exosomal microRNAs as predictive markers for chemoresistance in advanced colorectal cancer. *Cancer Chemother. Pharmacol.* 84, 315–325. doi: 10.1007/s00280-019-03867-6

Kaminskyy, V. O., Piskunova, T., Zborovskaya, I. B., Tchevkina, E. M., and Zhivotovsky, B. (2012). Suppression of basal autophagy reduces lung cancer cell proliferation and enhances caspase-dependent and -independent apoptosis by stimulating ROS formation. *Autophagy* 8, 1032–1044. doi: 10.4161/auto.20123

Kanemoto, S., Nitani, R., Murakami, T., Kaneko, M., Asada, R., Matsuhisa, K., et al. (2016). Multivesicular body formation enhancement and exosome release during endoplasmic reticulum stress. *Biochem. Biophys. Res. Commun.* 480, 166–172. doi: 10.1016/j.bbrc.2016.10.019

Kang, R., Zeh, H., Lotze, M., and Tang, D. (2020). The multifaceted effects of autophagy on the tumor microenvironment. *Adv. Exp. Med. Biol.* 1225, 99–114. doi: 10.1007/978-3-030-35727-6_7

Keulers, T. G., Schaaf, M. B. E., and Rouschop, K. M. A. (2016). Autophagy-dependent secretion: contribution to tumor progression. *Front. Oncol.* 25:251. doi: 10.3389/fonc.2016.00251

Kimura, T., Jia, J., Kumar, S., Choi, S. W., Gu, Y., Mudd, M., et al. (2017). Dedicated SNAREs and specialized TRIM cargo receptors mediate secretory autophagy. *EMBO J.* 36, 42–60. doi: 10.15252/embj.201695081

Kucharzewska, P., Christianson, H. C., Welch, J. E., Svensson, K. J., Fredlund, E., Ringnér, M., et al. (2013). Exosomes reflect the hypoxic status of glioma cells and mediate hypoxia-dependent activation of vascular cells during tumor development. *Proc. Natl. Acad. Sci. U.S.A.* 110, 7312–7317. doi: 10.1073/pnas.1220998110

Kulka, L. A. M., Fangmann, P. V., Panfilova, D., and Olzscha, H. (2020). Impact of HDAC inhibitors on protein quality control systems: consequences for precision medicine in malignant disease. *Front. Cell Dev. Biol.* 8:425. doi: 10.3389/fcell.2020.00425

Kulkarni, B., Gondaliya, P., Kirave, P., Rawal, R., Jain, A., Garg, R., et al. (2020). Exosome-mediated delivery of miR-30a sensitize cisplatin-resistant variant of oral squamous carcinoma cells via modulating Beclin1 and Bcl2. *Oncotarget* 11, 1832–1845. doi: 10.18632/oncotarget.27557

Kulshreshtha, R., Ferracin, M., Wojcik, S. E., Garzon, R., Alder, H., Agosto-Perez, F. J., et al. (2007). A microRNA signature of hypoxia. *Mol. Cell. Biol.* 27, 1859–1867. doi: 10.1128/MCB.01395-06

Kumar, A., and Deep, G. (2020). Hypoxia in tumor microenvironment regulates exosome biogenesis: molecular mechanisms and translational opportunities. *Cancer Lett.* 479, 23–30. doi: 10.1016/j.canlet.2020.03.017

Kumar, D., Gupta, D., Shankar, S., and Srivastava, R. K. (2014). Biomolecular characterization of exosomes released from cancer stem cells: possible implications for biomarker and treatment of cancer. *Oncotarget* 6, 3280–3291. doi: 10.18632/oncotarget.2462

Leidal, A. M., Huang, H. H., Marsh, T., Solvik, T., Zhang, D., Ye, J., et al. (2020). The LC3-conjugation machinery specifies the loading of RNA-binding proteins into extracellular vesicles. *Nat. Cell Biol.* 22, 187–199. doi: 10.1038/s41556-019-0450-y

Li, X. Q., Liu, J. T., Fan, L. L., Liu, Y., Cheng, L., Wang, F., et al. (2016). Exosomes derived from gefitinib-treated EGFR-mutant lung cancer cells alter cisplatin sensitivity via up-regulating autophagy. *Oncotarget* 7, 24585–24595. doi: 10.18632/oncotarget.8358

Lin, J., Lu, X., Liao, S., Chen, X., Wang, S., Zhao, C., et al. (2019). Cross-regulation between exosomal and autophagic pathways: promising therapy targets in disease. *Discov. Med.* 27, 201–210.

Liou, W., Geuze, H. J., Geelen, M. J. H., and Slot, J. W. (1997). The autophagic and endocytic pathways converge at the nascent autophagic vacuoles. *J. Cell Biol.* 136, 61–70. doi: 10.1083/jcb.136.1.61

Liu, D. X., Li, P. P., Guo, J. P., Li, L. L., Guo, B., Jiao, H. B., et al. (2019). Exosomes derived from HBV-associated liver cancer promote chemoresistance by upregulating chaperone-mediated autophagy. *Oncol. Lett.* 17, 323–331. doi: 10.3892/ol.2018.9584

Liu, Y., Yin, Z., Lu, P., Ma, Y., Luo, B., Xiang, L., et al. (2020). Lung carcinoma cells secrete exosomal MALAT1 to inhibit dendritic cell phagocytosis, inflammatory response, costimulatory molecule expression and promote dendritic cell autophagy via AKT/mTOR pathway. *Onco Targets Ther.* 13, 10693–10705. doi: 10.2147/OTT.S256669

Lu, J. F., Luk, F., Gong, J., Jaiswal, R., Grau, G. E., and Bebawy, M. (2013). Microparticles mediate MRP1 intercellular transfer and the re-templating of intrinsic resistance pathways. *Pharmacol. Res.* 76, 77–83. doi: 10.1016/j.phrs.2013.07.009

Luga, V., Zhang, L., Viloria-Petit, A. M., Ogunjimi, A. A., Inanlou, M. R., Chiu, E., et al. (2012). Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration. *Cell* 151, 1542–1556. doi: 10.1016/j.cell.2012.11.024

Malhotra, J., Jabbour, S., Orlick, M., Riedlinger, G., Guo, Y., White, E., et al. (2019). Phase Ib/II study of hydroxychloroquine in combination with chemotherapy in patients with metastatic non-small cell lung cancer (NSCLC). *Cancer Treat. Res. Commun.* 21:100158. doi: 10.1016/j.ctarc.2019.100158

Martinez, J., Almendinger, J., Oberst, A., Ness, R., Dillon, C. P., Fitzgerald, P., et al. (2011). Microtubule-associated protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. *Proc. Natl. Acad. Sci. U.S.A.* 108, 17396–17401. doi: 10.1073/pnas.1113421108

Matsuo, H., Chevallier, J., Mayran, N., Le Blanc, I., Ferguson, C., Fauré, J., et al. (2004). Role of LBPA and Alix in multivesicular liposome formation and endosome organization. *Science* 303, 531–534. doi: 10.1126/science.1092425

Mauthe, M., Orhon, I., Rocchi, C., Zhou, X., Luhr, M., Hijlkema, K. J., et al. (2018). Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. *Autophagy* 14, 1435–1455. doi: 10.1080/15548627.2018.1474314

Mazure, N. M., and Pouysségur, J. (2010). Hypoxia-induced autophagy: cell death or cell survival? *Curr. Opin. Cell Biol.* 22, 177–180. doi: 10.1016/j.ceb.2009.11.015

Meng, W., Hao, Y., He, C., Li, L., and Zhu, G. (2019). Exosome-orchestrated hypoxic tumor microenvironment. *Mol. Cancer* 18:57. doi: 10.1186/s12943-019-0982-6

Minakaki, G., Menges, S., Kittel, A., Emmanouilidou, E., Schaeffner, I., Barkovits, K., et al. (2018). Autophagy inhibition promotes SNCA/alpha-synuclein release and transfer via extracellular vesicles with a hybrid autophagosome-exosome-like phenotype. *Autophagy* 14, 98–119. doi: 10.1080/15548627.2017.1395992

Mizushima, N., Yoshimori, T., and Ohsumi, Y. (2011). The role of Atg proteins in autophagosome formation. *Annu. Rev. Cell Dev. Biol.* 27, 107–132. doi: 10.1146/annurev-cellbio-092910-154005

Mulcahy Levy, J. M., and Thorburn, A. (2020). Autophagy in cancer: moving from understanding mechanism to improving therapy responses in patients. *Cell Death Differ.* 27, 843–857. doi: 10.1038/s41418-019-0474-7

Murrow, L., Malhotra, R., and Debnath, J. (2015). ATG12–ATG3 interacts with Alix to promote basal autophagic flux and late endosome function. *Nat. Cell Biol.* 17, 300–310. doi: 10.1038/ncb3112

Nazio, F., Bordi, M., Cianfanelli, V., Locatelli, F., and Cecconi, F. (2019). Autophagy and cancer stem cells: molecular mechanisms and therapeutic applications. *Cell Death Differ.* 26, 690–702. doi: 10.1038/s41418-019-0292-y

Nishimura, T., and Tooze, S. A. (2020). Emerging roles of ATG proteins and membrane lipids in autophagosome formation. *Cell Discov.* 6:32. doi: 10.1038/s41421-020-0161-3

Parolini, I., Federici, C., Raggi, C., Lugini, L., Palleschi, S., De Milito, A., et al. (2009). Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J. Biol. Chem.* 284, 34211–34222. doi: 10.1074/jbc.M109.041152

Peinado, H., Aleèkoviæ, M., Lavotshkin, S., Matei, I., Costa-Silva, B., Moreno-Bueno, G., et al. (2012). Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat. Med.* 18, 883–891. doi: 10.1038/nm.2753

Ponpuak, M., Mandell, M. A., Kimura, T., Chauhan, S., Cleyrat, C., and Deretic, V. (2015). Secretory autophagy. *Curr. Opin. Cell Biol.* 35, 106–116. doi: 10.1016/j.ceb.2015.04.016

Rahbarghazi, R., Jabbari, N., Sani, N. A., Asghari, R., Salimi, L., Kalashani, S. A., et al. (2019). Tumor-derived extracellular vesicles: reliable tools for Cancer diagnosis and clinical applications. *Cell Commun. Signal.* 17:73. doi: 10.1186/s12964-019-0390-y

Raposo, G., and Stoorvogel, W. (2013). Extracellular vesicles: exosomes, microvesicles, and friends. *J. Cell Biol.* 200, 373–383. doi: 10.1083/jcb.201211138

Rezaie, J., Ajezi, S., Avci, C. B., Karimipour, M., Geranmayeh, M. H., Nourazarian, A., et al. (2018). Exosomes and their application in biomedical field: difficulties and advantages. *Mol. Neurobiol.* 55, 3372–3393. doi: 10.1007/s12035-017-0582-7

Rodríguez-Perálvarez, M., Guerrero, M., Barrera, L., Ferrín, G., Álamo, J. M., Ayllón, M. D., et al. (2018). Impact of early initiated everolimus on the recurrence of hepatocellular carcinoma after liver transplantation. *Transplantation* 102, 2056–2064. doi: 10.1097/TP.0000000000002270

Salimi, L., Akbari, A., Jabbari, N., Mojarad, B., Vahhabi, A., Szafert, S., et al. (2020). Synergies in exosomes and autophagy pathways for cellular homeostasis and metastasis of tumor cells. *Cell Biosci.* 10:64. doi: 10.1186/s13578-020-00426-y

Santos, P., and Almeida, F. (2020). Role of exosomal miRNAs and the tumor microenvironment in drug resistance. *Cells* 9:1450. doi: 10.3390/cells9061450

Savina, A., Furlán, M., Vidal, M., and Colombo, M. I. (2003). Exosome release is regulated by a calcium-dependent mechanism in K562 cells. *J. Biol. Chem.* 278, 20083–20090. doi: 10.1074/jbc.M301642200

Schuh, A. L., and Audhya, A. (2014). The ESCRT machinery: from the plasma membrane to endosomes and back again. *Crit. Rev. Biochem. Mol. Biol.* 49, 242–261. doi: 10.3109/10409238.2014.881777

Scita, G., and Di Fiore, P. P. (2010). The endocytic matrix. *Nature* 463, 464–473. doi: 10.1038/nature08910

Shao, N., Xue, L., Wang, R., Luo, K., Zhi, F., and Lan, Q. (2019). miR-454-3p is an exosomal biomarker and functions as a tumor suppressor in glioma. *Mol. Cancer Ther.* 18, 459–469. doi: 10.1158/1535-7163.mct-18-0725

Singh, S. S., Vats, S., Chia, A. Y. Q., Tan, T. Z., Han, M. S., Ravi, M., et al. (2018). Dual role of autophagy in hallmarks of cancer. *Oncogene* 37, 1142–1158. doi: 10.1038/s41388-017-0046-6

Skog, J., Würdinger, T., van Rijn, S., Meijer, D. H., Gainche, L., Sena-Esteves, M., et al. (2008). Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat. Cell Biol.* 10, 1470–1476. doi: 10.1038/ncb1800

Steinbichler, T. B., Dudás, J., Skvortsov, S., Ganswindt, U., Riechelmann, H., and Skvortsova, I. I. (2019). Therapy resistance mediated by exosomes. *Mol. Cancer* 18:58. doi: 10.1186/s12943-019-0970-x

Stuffers, S., Sem Wegner, C., Stenmark, H., and Brech, A. (2009). Multivesicular endosome biogenesis in the absence of ESCRTs. *Traffic* 10, 925–937. doi: 10.1111/j.1600-0854.2009.00920.x

Sung, J. S., Kang, C. W., Kang, S., Jang, Y., Chae, Y. C., Kim, B. G., et al. (2020). ITGB4-mediated metabolic reprogramming of cancer-associated fibroblasts. *Oncogene* 39, 664–676. doi: 10.1038/s41388-019-1014-0

Svensson, K. J., Christianson, H. C., Wittrup, A., Bourseau-Guilmain, E., Lindqvist, E., Svensson, L. M., et al. (2013). Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated by caveolin-1. *J. Biol. Chem.* 288, 17713–17724. doi: 10.1074/jbc.M112.445403

Tan, H. W. S., Sim, A. Y. L., and Long, Y. C. (2017). Glutamine metabolism regulates autophagy-dependent mTORC1 reactivation during amino acid starvation. *Nat. Commun.* 8:338. doi: 10.1038/s41467-017-00369-y

Théry, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R., et al. (2018). Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J. Extracell. Vesicles* 7:1535750. doi: 10.1080/20013078.2018.1535750

Tian, J., Popal, M. S., Zhao, Y., Liu, Y., Chen, K., and Liu, Y. (2019). Interplay between exosomes and autophagy in cardiovascular diseases: novel promising target for diagnostic and therapeutic application. *Aging Dis.* 10, 1302–1310. doi: 10.14336/AD.2018.1020

Tian, T., Zhu, Y. L., Zhou, Y. Y., Liang, G. F., Wang, Y. Y., Hu, F. H., et al. (2014). Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery. *J. Biol. Chem.* 289, 22258–22267. doi: 10.1074/jbc.M114.588046

van Niel, G., Porto-Carreiro, I., Simoes, S., and Raposo, G. (2006). Exosomes: a common pathway for a specialized function. *J Biochem.* 140, 13–21. doi: 10.1093/jb/mvj128

Verfaillie, T., Salazar, M., Velasco, G., and Agostinis, P. (2010). Linking ER stress to autophagy: potential implications for cancer therapy. *Int J. Cell Biol.* 2010:930509. doi: 10.1155/2010/930509

Villarroya-Beltri, C., Baixauli, F., Mittelbrunn, M., Fernaìndez-Delgado, I., Torralba, D., Moreno-Gonzalo, O., et al. (2016). ISGylation controls exosome secretion by promoting lysosomal degradation of MVB proteins. *Nat. Commun.* 7:13588. doi: 10.1038/ncomms13588

Wang, B., Mao, J. H., Wang, B. Y., Wang, L. X., Wen, H. Y., Xu, L. J., et al. (2020). Exosomal miR-1910-3p promotes proliferation, metastasis, and autophagy of breast cancer cells by targeting MTMR3 and activating the NF-κB signaling pathway. *Cancer Lett.* 489, 87–99. doi: 10.1016/j.canlet.2020.05.038

Wang, X., Yin, X., and Yang, Y. (2019). Rasal2 suppresses breast cancer cell proliferation modulated by secretory autophagy. *Mol. Cell. Biochem.* 462, 115–122. doi: 10.1007/s11010-019-03615-7

White, E. (2015). The role for autophagy in cancer. *J. Clin. Invest.* 125, 42–46. doi: 10.1172/JCI73941

White, E., Karp, C., Strohecker, A. M., Guo, Y., and Mathew, R. (2010). Role of autophagy in suppression of inflammation and cancer. *Curr. Opin. Cell Biol.* 212–217. doi: 10.1016/j.ceb.2009.12.008

Wu, Z., He, D., and Li, H. (2020). Bioglass enhances the production of exosomes and improves their capability of promoting vascularization. *Bioact. Mater.* 6, 823–835. doi: 10.1016/j.bioactmat.2020.09.011

Wu, X., Zhou, Z., Xu, S., Liao, C., Chen, X., Li, B., et al. (2020). Extracellular vesicle packaged LMP1-activated fibroblasts promote tumor progression via autophagy and stroma-tumor metabolism coupling. *Cancer Lett.* 478, 93–106. doi: 10.1016/j.canlet.2020.03.004

Xu, J., Camfield, R., and Gorski, S. M. (2018). The interplay between exosomes and autophagy – partners in crime. *J. Cell Sci.* 131:jcs215210. doi: 10.1242/jcs.215210

Yang, Y., and Klionsky, D. J. (2020). Autophagy and disease: unanswered questions. *Cell Death Differ.* 27, 858–871. doi: 10.1038/s41418-019-0480-9

Yao, W., Guo, P., Mu, Q., and Wang, Y. (2020). Exosome-derived Circ-PVT1 contributes to cisplatin resistance by regulating autophagy, invasion, and apoptosis Via miR-30a-5p/YAP1 axis in gastric cancer Cells. *Cancer Biother. Radiopharm.* doi: 10.1089/cbr.2020.3578 [Epub ahead of print].

Yeon, M., Lee, S., Lee, J. E., Jung, H. S., Kim, Y., and Jeoung, D. (2019). CAGE-miR-140-5p-Wnt1 axis regulates autophagic flux, tumorigenic potential of mouse colon cancer cells and cellular interactions mediated by exosomes. *Front. Oncol.* 9:1240. doi: 10.3389/fonc.2019.01240

Yu, L., Chen, Y., and Tooze, S. A. (2018). Autophagy pathway: Cellular and molecular mechanisms. *Autophagy* 14, 207–215. doi: 10.1080/15548627.2017.1378838

Yu, S., Liu, C., Su, K., Wang, J., Liu, Y., Zhang, L., et al. (2007). Tumor exosomes inhibit differentiation of bone marrow dendritic cells. *J. Immunol.* 178, 6867–6875. doi: 10.4049/jimmunol.178.11.6867

Yun, C. W., and Lee, S. H. (2018). The roles of autophagy in cancer. *Int. J. Mol. Sci.* 19:3466. doi: 10.3390/ijms19113466

Yuwen, D., Ma, Y., Wang, D., Gao, J., Li, X., Xue, W., et al. (2019). Prognostic role of circulating exosomal miR-425-3p for the response of NSCLC to platinum-based chemotherapy. *Cancer Epidemiol. Biomarkers Prev.* 28, 163–173. doi: 10.1158/1055-9965.epi-18-0569

Zeng, Y., Yao, X., Liu, X., He, X., Li, L., Liu, X., et al. (2019). Anti-angiogenesis triggers exosomes release from endothelial cells to promote tumor vasculogenesis. *J. Extracell. Vesicles* 8:1629865. doi: 10.1080/20013078.2019.1629865

Zhang, M., Kenny, S. J., Xu, K., and Schekman, R. (2015). Translocation of interleukin-1β into a vesicle intermediate in autophagy-mediated secretion. *eLife* 4:e11205. doi: 10.7554/eLife.11205

Zhang, X., Shi, H., Yuan, X., Jiang, P., Qian, H., and Xu, W. (2018). Tumor-derived exosomes induce N2 polarization of neutrophils to promote gastric cancer cell migration. *Mol. Cancer* 17:146. doi: 10.1186/s12943-018-0898-6

Zhang, X., Wang, S., Wang, H., Cao, J., Huang, X., Chen, Z., et al. (2019). Circular RNA circNRIP1 acts as a microRNA-149-5p sponge to promote gastric cancer progression via the AKT1/mTOR pathway. *Mol. Cancer* 18:20. doi: 10.1186/s12943-018-0935-5

Zhao, Y. G., and Zhang, H. (2019). Autophagosome maturation: an epic journey from the ER to lysosomes. *J. Cell Biol.* 218, 757–770. doi: 10.1083/jcb.201810099

Zheng, K., Ma, J., Wang, Y., He, Z., and Deng, K. (2020). Sulforaphane inhibits autophagy and induces exosome-mediated paracrine senescence via regulating mTOR/TFE3. *Mol. Nutr. Food Res.* 64:e1901231. doi: 10.1002/mnfr.201901231

Zhou, J., Hu, S. E., Tan, S. H., Cao, R., Chen, Y., Xia, D., et al. (2012). Andrographolide sensitizes cisplatin-induced apoptosis via suppression of autophagosome-lysosome fusion in human cancer cells. *Autophagy* 8, 338–349. doi: 10.4161/auto.18721

Zhou, T., Lin, D., Chen, Y., Peng, S., Jing, X., Lei, M., et al. (2019). α-synuclein accumulation in SH-SY5Y cell impairs autophagy in microglia by exosomes overloading miR-19a-3p. *Epigenomics* 11, 1661–1677. doi: 10.2217/epi-2019-0222

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Colletti, Ceglie, Di Giannatale and Nazio. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Oncology

REVIEW
published: 20 January 2021
doi: 10.3389/fonc.2020.610472

Autophagy Regulation and Photodynamic Therapy: Insights to Improve Outcomes of Cancer Treatment

Waleska K. Martins[1], Renata Belotto[2], Maryana N. Silva[1], Daniel Grasso[3], Maynne D. Suriani[4], Tayná S. Lavor[4], Rosangela Itri[5], Mauricio S. Baptista[6] and Tayana M. Tsubone[4]**

[1] Laboratory of Cell and Membrane, Anhanguera University of São Paulo, São Paulo, Brazil, [2] Perola Byington Hospital Gynecology - Lasertherapy Clinical Research Department, São Paulo, Brazil, [3] CONICET, Instituto de Estudios de la Inmunidad Humoral (IDEHU), Universidad de Buenos Aires, Buenos Aires, Argentina, [4] Institute of Chemistry, Federal University of Uberlândia, Uberlândia, Brazil, [5] Institute of Physics, University of São Paulo, São Paulo, Brazil, [6] Institute of Chemistry, University of São Paulo, São Paulo, Brazil

OPEN ACCESS

Edited by:
Boris Zhivotovsky, Karolinska Institutet (KI), Sweden

Reviewed by:
Dmitri V. Krysko, Ghent University, Belgium
Luciana Dini, Sapienza University of Rome, Italy

***Correspondence:**
Waleska K. Martins
wkerllenmartins@gmail.com
Tayana M. Tsubone
tayana.tsubone@ufu.br

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *25 September 2020*
Accepted: *03 December 2020*
Published: *20 January 2021*

Citation:
Martins WK, Belotto R, Silva MN, Grasso D, Suriani MD, Lavor TS, Itri R, Baptista MS and Tsubone TM (2021) Autophagy Regulation and Photodynamic Therapy: Insights to Improve Outcomes of Cancer Treatment. Front. Oncol. 10:610472. doi: 10.3389/fonc.2020.610472

Cancer is considered an age-related disease that, over the next 10 years, will become the most prevalent health problem worldwide. Although cancer therapy has remarkably improved in the last few decades, novel treatment concepts are needed to defeat this disease. Photodynamic Therapy (PDT) signalize a pathway to treat and manage several types of cancer. Over the past three decades, new light sources and photosensitizers (PS) have been developed to be applied in PDT. Nevertheless, there is a lack of knowledge to explain the main biochemical routes needed to trigger regulated cell death mechanisms, affecting, considerably, the scope of the PDT. Although autophagy modulation is being raised as an interesting strategy to be used in cancer therapy, the main aspects referring to the autophagy role over cell succumbing PDT-photoinduced damage remain elusive. Several reports emphasize cytoprotective autophagy, as an ultimate attempt of cells to cope with the photo-induced stress and to survive. Moreover, other underlying molecular mechanisms that evoke PDT-resistance of tumor cells were considered. We reviewed the paradigm about the PDT-regulated cell death mechanisms that involve autophagic impairment or boosted activation. To comprise the autophagy-targeted PDT-protocols to treat cancer, it was underlined those that alleviate or intensify PDT-resistance of tumor cells. Thereby, this review provides insights into the mechanisms by which PDT can be used to modulate autophagy and emphasizes how this field represents a promising therapeutic strategy for cancer treatment.

Keywords: photodynamic therapy, autophagy, cell death, cancer, clinical trials

INTRODUCTION

Cancer remains one of the most common causes of health problems worldwide with increasing rates in developed and under-developed/developing countries (1). The new cases and deaths numbers were estimated at 18.1 and 9.6 million, respectively, according to GLOBOCAN updates (2). Cancer more often affects aged people (50.8% of cases), but there is a worldwide concern about those >65 years in the near future (3, 4). Over the next 10 years, people will suffer more death from cancer than from other very common diseases, such as diabetes (5). It is clear, therefore, that although cancer treatment has considerably improved in the last decades, the fight against this disease is in urgent need of novel tools.

Cancer is a multifactorial disease and despite the many recently introduced chemo and immunotherapies the general clinical outcome and prognosis of cancer patients is not optimistic at all. Overall, novel therapies are less detrimental to the individual because they are specific in modulating different immune/biochemical pro-death modes (e.g. apoptosis), to get rid of tumor cells. Unfortunately, by a phenomenon known as chemo-adaptation and dormancy many human cancers (e.g. cutaneous melanoma, breast, head, and neck tumors) can downregulate specifically the pro-apoptotic mechanisms, worsening the outcome and the prognosis of cancer patients. In addition to proliferation and plasticity abilities, tumor cells considered "*stemness*" gradually give rise to chemoresistance *via* a distinct variety of mechanisms and pathways. For this reason, the modulation of different cell death pathways could help to define complementary or alternative strategies to those based on the activation of apoptosis.

Since all cells have membranes whose integrity is necessary for survival, therapeutic strategies that address specific oxidative damage in the membranes of organelles have great potential to avoid therapeutic resistance. Photodynamic Therapy (PDT) is a non-invasive and efficient strategy based on photophysical principles that may provide specific oxidative damage in organelles such as the endoplasmic reticulum, mitochondria, and lysosomes. Herein, we present our current knowledge regarding tumor resistance concerning the suppression of autophagic response, in an attempt to improve clinical outcomes. In this scenery, the photo-mediated pro-death autophagy emphasizes PDT as a promising therapy to deal with tumors that evade apoptosis. Undeniably, PDT has been applied with success to treat several types of human cancers with tolerable side effects. However, as PDT-resistance has increased due to distinct reasons (oxidative-scavenger response, autophagy activation, drug extrusion, and others), we will discuss the pitfalls and successes of its use, considering autophagy as a therapeutic target to improve tumor remission. Considering the PDT photophysics and photochemistry effects, as well as the photooxidative-mediated membrane damage, we will discuss the molecular mechanism for tumor-resistance, particularly focusing on the biological, molecular, and translational aspects of the PDT-related cancer treatments.

PHOTODYNAMIC THERAPY (PDT)

Considering the difficulties and challenges in conventional cancer treatment, such as tumor resistance, new treatment concepts for both primary care and adjuvant therapy are highly necessary. PDT is a well-established medical procedure due to the selective cancer eradication (sparing normal cells), especially when tumor sites can be demarcated (6). The PDT advantages compared to the conventional cancer treatments include: (i) it does not seem to induce drug resistance, (ii) promote selective cancer destruction, preserving the surrounding normal tissues (iii) preserving the native tissue architecture and giving a decisively better recovery compared with surgery (iv) can be used with other therapies (7).

PDT is definitively less invasive compared to surgery, and more precise than chemotherapy and, finally, as opposed to radiotherapy, may be repeated several times (8). A photosensitizer (PS) molecule can be administered intravenously, intraperitoneally, or topically to

Abbreviations: 1400W, N-[3- (aminomethyl)benzyl]acetamidine; 2-DG, 2-deoxy-D-glucose; 3-MA, 3-methyladenine; 5-ALA, 5-aminolevulinic acid; ABC, ATP binding cassette; ABCB1, ATP Binding Cassette Subfamily B Member 1 or P-Glycoprotein 1; ABCB6, ATP Binding Cassette Subfamily B Member 6 (Langereis Blood Group); ABCG2, ATP Binding Cassette Subfamily G Member 2 (Junior Blood Group) or BCRP; AKT, Ser and Thr kinase AKT, also known as protein kinase B; APAF-1, Apoptotic protease-activating factor-1; ATF4, Activating transcription factor 4; ATF6, Activating transcription factor 6; BAF-A1, Bafilomycin A1; BAK, Bcl-2 homologous antagonist killer; BAX, Bcl-2 -associated X protein; BLC-2, BCL2 apoptosis regulator; BCL-xL, BCL2 Like 1; BID, BH3 interacting-domain death agonist; BPD, Benzoporphyrin or verteporfin; Ce6, Chlorin e6; CHOP, C/EBP homologous protein or DNA damage inducible transcript 3 (DDIT3); CisDiMPyP, *meso-cis*-di(N-methyl-4-pyridyl)diphenyl porphyrin dichloride; cPTIO, 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; CQ, Chloroquine; DCA, Dichloroacetate; dc-IR825, Near-infrared cyanine dye; DMMB, 1,9 dimethylmethylene blue; eIF2α, Eukaryotic translation initiation factor 2A; EGFR, Epidermal growth factor receptor; ER, Endoplasmic reticulum; ERK, Extracellular signal-regulated kinases; H_2O_2, Hydrogen peroxide; HAL, Hexaminolevulinate or 5-Aminolevulinic acid hexyl ester; HPPH, 2-(1-Hexyloxyethyl)-2-devinyl pyropheophorbide-a; HSP27, Heat shock protein 27; HSP70, Heat Shock Protein Family A (Hsp70) Member 4; HSP90, Heat shock Protein 90 Alpha Family Class A Member 1; HSPA5, Heat Shock Protein Family A (Hsp70) Member 5; iNOS/NOS2, Inducible nitric oxide synthase; JNK, c-jun N-terminal kinases; LAMP2A, Lysosome-associated membrane protein type 2A; LED, Light-emitting diode; MAP2K1, Mitogen-activated protein kinase kinase 1; MAPK1/ERK2, Mitogen-activated protein kinase 1; MAPK3/ERK1, Mitogen-activated protein kinase 3; MPPa, Pyropheophorbide-a methyl ester; m-THPC, *meta*-tetrahydroxyphenylchlorin; mTOR, Mammalian target of rapamycin; mTORC1, mTOR complex 1; NPe6, Mono-L-aspartyl chlorin e6 or talaporfin sodium; N-TiO_2, Nitrogen-doped titanium dioxide; $^{\bullet}OH$, Hydroxyl radical; ($O_2^{\bullet-}$), Superoxide ion; $1O_2$, Singlet oxygen; p38α, P38 mitogen-activated protein kinase or Mitogen-Activated Protein Kinase 14; PARP1, Poly(ADP-ribose) polymerase 1; Pc13, Zinc(II) phthalocyanine Pc13; PEPT1, Oligopeptide transporter peptide transporter 1; PFKFB3, 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3; PpIX, Protoporphyrin IX; PS, Photosensitizer; PSA, Pepstatin A; RIPK3, Receptor Interacting Serine/Threonine Kinase 3; ROS, Reactive oxygen species; SLC15A1, Solute Carrier Family 15 Member 1 or PEPT1; TPGS, D-α-tocopheryl polyethylene glycol 1000 succinate; TPPOH, 5-(4-hydroxyphenyl)-10,15,20-triphenylporphyrin; TPPOH-X SNPs, Silica nanoparticles (SNPs) coated with xylan-TPPOH conjugate (TPPOH-X); TPPS, Tetraphenylporphinesulfonate; $TPCS_{2a}$, Disulfonated tetraphenyl chlorine; $TPPS_{2a}$, *meso*-Tetraphenylporphine disulphonic acid disodium salt (adjacent isomer), WST11, Palladium bacteriopheophorbide.; TRIB3, Tribbles-related protein 3; ULK1, Unc51-like autophagy activating kinase 1; VMP1, Vacuole membrane protein 1.

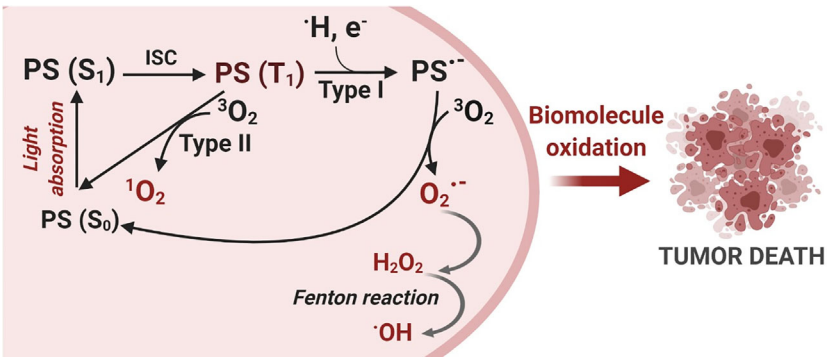


FIGURE 1 | Photodynamic Therapy Mechanism. The photosensitization process starts with a photon absorption that converts the photosensitizer PS (S_0) ground state to a more energetic state known as a singlet excited state PS (S_1). Then, an intersystem crossing conversion (ISC) changes the PS multiplicity to a triplet excited state PS (T_1). PS (T_1) can interact with molecules nearby and react *via* two distinct mechanisms: Type I – electron transfer and Type II – energy transfer, generating reactive oxygen species (ROS). Finally, oxidative species damage biomolecules and can trigger cell death. Created with BioRender.com.

the patient, and the tumors tissue sites are selectively irradiated. Although these components (i.e., PS and light) are harmless alone, when combined they provide localized antitumor therapy. This avoids damage to healthy cells thus preventing side effects. The combination of PS and light results in the generation of reactive excited states (singlet and triplet excited states) as well as several reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), hydroxyl radical ($^{\cdot}OH$), superoxide ion ($O_2^{\cdot-}$), and hydrogen peroxide (H_2O_2). These reactive species can efficiently oxidize and irreversibly damage targeted tumor tissues/cells (9–11).

Light with a specific wavelength PS triggers the photooxidative process, as summarized in **Figure 1**. PS excitation through photon absorption transforms the ground state PS (S_0) into an excited state - singlet excited state PS (S_1). Next, PS (S_1) can be converted into a triplet excited state (PS (T_1), by the change in the spin of electron *via* a process known as intersystem crossing (ISC). Due to its new spin configuration, PS (T_1) can live long enough to interact with species nearby, resulting in two main photosensitization mechanisms: (a) energy transfer to oxygen (Type II process) or (b) a directed reaction with biological substrates (Type I process). On the Type II process, energy transfer to molecular oxygen ($^3O_2$) yields the highly reactive oxygen state known as singlet oxygen ($^1O_2$), an electrophilic molecule that is often considered the main PDT performance species (10–12). Type I processes are based on reactions between PS (T_1) and nearby biomolecules, forming a variety of products, which can start a radical chain reaction. The free radicals generated during the Type I mechanism can still react with oxygen, resulting in the production of ROS such as $\cdot OH$, ($O_2^{\cdot-}$), and H_2O_2 (10–12).

These two reaction mechanisms, Type I and Type II, invariably involve oxygen as either a primary or a secondary intermediate reactant and are also called photosensitized oxidation reactions (11, 13). Both mechanisms may occur simultaneously, and a balance between them is important for ROS production and, in turn, determines the overall photo-cytotoxicity effectiveness of the PDT reaction (11, 14). The dominant mechanism will depend on the PS itself, the type of substrate, the distance between the PS and the oxidative targets as well as the oxygen concentration.

The PDT efficiency depends on the illumination conditions, the chemical properties, and the intra-tumoral localization of the PSs localization. Selecting a suitable device for the tumor region irradiation is a fundamental factor in PDT protocols. The main types of light sources used in PDT include lasers, light-emitting diodes (LEDs), and lamps. Each category source presents advantages and disadvantages. For this reason, a choice of proper light source needs to be carefully evaluated according to the PS, tumor location, and the light dose to be delivered (15). The geometry of the tumor area which, sometimes, is not easy to access, determines the decision of the correct light apparatus to be used (15). As an example of selecting a suitable light device, Davanzo et al. demonstrated that it is possible to obtain different PDT outcomes depending on the light source used (16). Under the same light dose exposure, they reported that continuous laser was a better light source compared to other devices (pulsed laser and LED) under the same light dose exposure (16). Indeed, other factors impact the final PDT outcome, including the amount and the type of reactive species, which is highly dependent on the photochemical and photophysical properties of the PS.

Several classes of PSs have been commonly employed in PDT, including porphyrins, chlorins, phthalocyanines, and phenothiazines (10). Each one of them presents distinct advantages and disadvantages regarding the chromophore type. For example, Photofrin™ (porfimer sodium), which is an oligomer and was the first PS approved by the FDA for the treatment of bladder cancer in Canada in 1993 (17). Its structure is not well defined, but its aqueous suspension can be applied intravenously. However, the absorption in the low 'therapeutic window' (between 600-800 nm) and a prolonged (~ 4 weeks) skin photosensitivity is an important side effect (17). On the other hand, phthalocyanines have a high molar absorption coefficient in the red spectral region but are not water-soluble. To deal with this limitation, a liposomal zinc phthalocyanine was developed and has been tested in phase 1 or 2 clinical trials for

solid tumors (17). However, it did not reach the clinical practice, probably due to issues concerning the stability and the difficulty of large-scale liposome (18).

Since several ROS species have high reactivity, short lifetimes, and consequently small diffusion pathways (12), only those PSs nearby to the biological substrates can cause tumor photodamage (12, 19). $^1O_2$ lifetime in pure water is ~4 μs (20), which provides a mean diffusion distance traveled by $^1O_2$ molecules in water of less than 200 nm, without considering any other reaction with biomolecules besides its intrinsic decay. Although the average dimensions of mammalian cells are around 10-30 μm diameter, the $^1O_2$ mediated oxidative-damage would reach only short distances, reaching specifically a target PDT organelle (12, 21). Therefore, generating amounts of ROS does not mean PS effectiveness (22). If PS is near to an intracellular target, the photo-generated ROS would oxidize biomolecules in a more specific way (22). Thus, the oxidative reactions primarily affect only nearby PS-targeted organelles (12). Also, the relative oxygen concentration may favor or disfavor $^1O_2$ formation, which may amplify (or not) the biomolecule's oxidation reactions (23, 24). Another parameter that drives PDT efficiency is the molecular structure of the PS. Of note, PSs belonging to the same class may have distinct properties, given the diversity of the side-groups that can be attached to the lead chromophore (25–31).

Knowing that biological membranes guarantee cell homeostasis due to their crucial role in compartmentalizing intracellular content and organelles, they are particularly important targets for PDT. The basic structural membrane elements are the lipid bilayer and the integral or linked proteins (32, 33). Both the lipids and the integral proteins display amphiphilic characteristics, explaining why PSs that exhibit amphipathic character will interact with the membrane, independent of whether it is a plasma, mitochondrial, lysosomal, or endoplasmic reticulum membrane. As an example, Engelman et al. compared porphyrins with two charged groups around the ring at position *cis* and *trans* and observed that *cis*-isomer presented a much larger binding to the membrane than predicted by water/octanol partition (log P_{OW}) (34). This is because the cis-isomer has an optimized amphiphilic structure that matches the amphiphilic structures of the lipids in the bilayer. As a result, an enhanced photodynamic efficiency was perceived regardless of the type of the membrane (i.e., liposomes, mitochondria, and erythrocytes membrane) (34). Tsubone et al. also studied a series of amphiphilic PSs displaying opposite charges (negative or positive) and noticed that hydrophobic and dipolar interactions play crucial roles in defining the affinity of these molecules to membranes (12, 35). Although the increase in the alkyl chain length above certain limits leads to aggregation and decreases in the PS photoactivity, increasing the hydrophobicity up to certain limits has also been associated with enhanced cell photokilling efficiency (36–38). Another parameter that favors the PS binding in the lipid membranes is the molecule asymmetry. In Porphyrin, a peripherical group at *meta*-position was found to be more phototoxic than its *para*-isomer, mainly because the *meta*-isomer asymmetry favors the PS-membrane interaction compared to the symmetric *para-isomer* (39).

Because proteins are the most abundant biomolecules in cells, they probably act as major targets for photo-oxidation (40, 41). The main forces that govern PS-protein interaction are well described in the literature (42, 43). Phototoxic outcomes seem to depend on PS-protein interaction. Towards this end, Cozzolino et al. bound curcumin to bovine serum albumin and showed that the conjugate displays a better photodynamic effect when compared to the unbound curcumin (44). Proteins can also be used as PS carriers. Recently, it was reported a macromolecular approach of a synergistic combination of Ru-complexes on a protein carrier with subcellular mitochondria targeting groups, allows enhanced phototoxicity and efficacy (45).

Linking the PS to a monoclonal antibody allows the photodamage to be addressed to key specific molecular markers, present, for example in tumor surface. In this context, the epidermal growth factor receptor (EGFR) is a promising target for PS-immunoconjugates, considering it is commonly overexpressed in cancer cells (46). Indeed, it has been recently shown that the verteporfin-immunoconjugate (monoclonal antibody targeting EGFR) causes significantly higher levels of cell death in ovarian metastatic cancer cells (overexpressing the EGFR receptor) compared to the cell death without EGFR overexpression (47, 48). Besides the cell-surface EGFR receptors that have antibodies, such as cetuximab (49) or panitumumab (50), recent reports pointed out as promising targets in preclinical models (51) the photobiomodulation of tumor-associated regulatory T cells (52, 53).

Cellular compartments vary substantially and the photosensitizer structures determine the subcellular location of the photodamage and control cell death efficiency (12). Therefore, understanding the cellular and molecular photodynamic mechanisms can lead to an optimization in the PDT efficacy. As long as each PS has a distinctive subcellular localization profile, the PDT-mediated cell death can be modulated regarding specific oxidative stress in the targeted organelle (47). For instance, whereas CisDiMPyP incorporates into mitochondria, $TPPS_{2a}$ accumulates mainly within lysosomes (35). Other PSs can evoke mitochondrial, lysosomal, and/or ER photodamage (35, 54–62). Such a possibility of PSs selectively inducing damage in targeted organelles is key to potentiate the photo-induced cell death (63, 64).

MOLECULAR MECHANISMS FOR TUMOR RESISTANCE TO PDT

The resistance to regulated cell death mechanisms (RCD) is one of the most prominent cancer hallmarks, intrinsically contributing to tumor recurrence and metastasis. Accordingly, the tumor relapse to current conventional chemotherapies has increased up to 2500-fold (65). The PDT approach (i.e., light energy and PS concentration) might eliminate most of the tumor cells, however, some of them may elicit their survival and dormancy, leading to phototherapeutic cancer resistance. Thus, despite PDT potentially circumventing cancer recurrence to some chemotherapies (e.g. cisplatin, dacarbazine, or 5-Fluoracil)

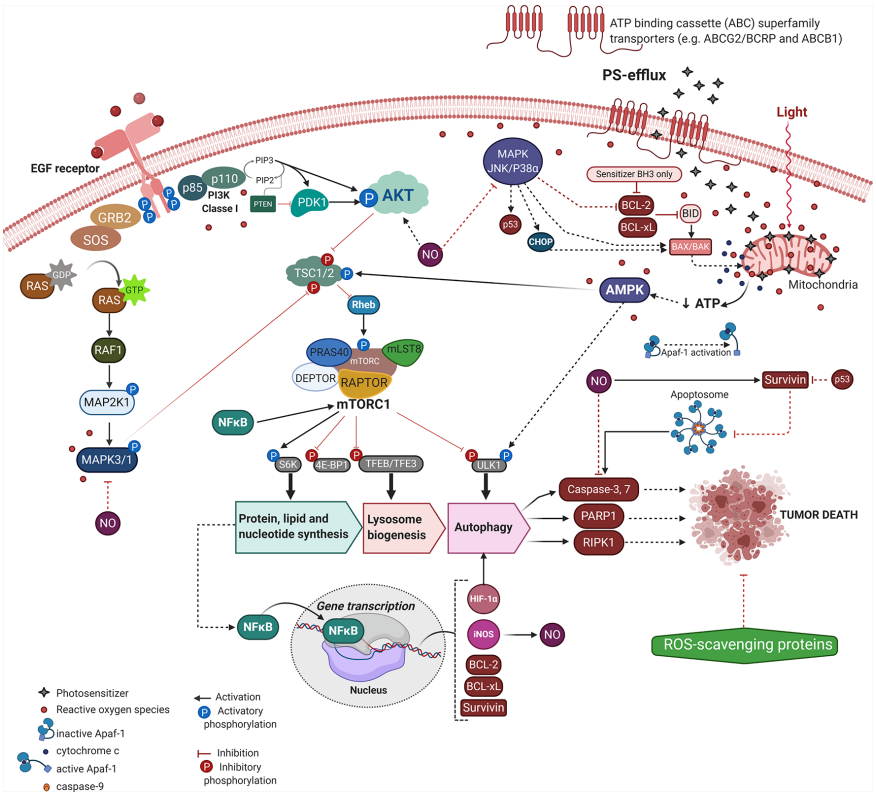


FIGURE 2 | Molecular mechanisms underlying PDT-resistance of tumor cells. Created with BioRender.com.

(66–68), its promisor tumor dealing potential might also fail (69–72). Therefore, just as with other approaches similar to chemotherapy and radiotherapy, post-PDT treatment tumors are prone to become resistant and more aggressive (73, 74). Most of these mechanisms that elicit tumor PDT-resistance relies on the number of phototherapy sessions, the cell type, delivery system, and photo-physical aspects of the PS (65, 74–76). Although the PDT resistance mechanism remains elusive, we briefly consider the main molecular mechanisms underlying the tumor defense against the photooxidative damage and PS uptake (**Figure 2**). We also pointed out the new PDT approaches to deal with tumor recurrence and maximize the phototherapeutic efficacy.

Tumoral tissues might acquire an intrinsic resistance to treatment or activate alternative compensatory signaling pathways to handle cytotoxicity (73). Indeed, most of those resistant mechanisms comprise an adaptative response to the therapeutic-mediated extrinsic stresses, including mutations, altered genetic and epigenetic profiles, dysregulation of regulatory proteins of apoptosis or autophagy, dormancy, surrogation of the chemotherapeutic targets, drug efflux capacity, and stimulation of compensatory signaling or mediated repair pathways (76–80). Moreover, we can focus on tumor relapse related to the tumoral microenvironment, limited incorporation of the PS, hypoxia, and low penetration of radiation into tumoral mass (73).

In many cases, cell morphology, cytoskeleton, and cell adhesion changes have been observed in cells or tissue under photosensitization, which was correlated to significant impairment of migratory and invasive behaviors (81). The photo-mediated alterations into cytoskeleton (e.g. shorter stress fibers, decreased number of dorsal fibers, loss of cell-to-cell interactions, and epithelial morphology) ascribes less invasiveness and migratory properties to tumor cells, which lead to higher cellular plasticity and PDT resilience (81). Paradoxically, cytoskeleton alteration associated with invasion and metastasis might result in PDT-resistance (81). Such discrepancies can occur due to higher tumor heterogeneity, as well as the presence of hypoxic regions that may impair the PDT-overcome in innumerous ways beyond the limitation of oxygen, one of the components of phototherapy (**Figure 1**).

In an elegant model using heterotypic spheroids composed of human colorectal SW480 cancer cells and fibroblast, Lamberti et al. demonstrated that the tumor-stroma interaction with a hypoxic environment significantly impairs the 5-ALA metabolism, and so reduces the production of the endogenous PpIX (Protoporphyrin IX), the photosensitizer molecule (82). In this context of oxygen deprivation, HIF-1α is the key player and, despite conferring adaptability to hypoxia, it might also assign resistance to PDT by at least avoiding intracellular PS accumulation. Additionally, the HIF-1α mediated resistance

could be induced by the PDT itself (83). In colorectal cancer cell spheroids, the PpIX-PDT can activate the MAPK1/ERK2 and MAPK3/ERK1 pathway as an adaptative and survival mode to resist the mitochondrial photooxidative damage. This molecular event results in the transcriptional activation of HIF-1α, suggesting that the ROS-MAPK1/3-HIF-1α axis may be a solution for PDT-resistance (83). It is worth noting that autophagy induction in response to PDT might be also related to HIF-1α. The simple HIF-1α stabilization induces autophagy in colon Caco-2 and SW480 cancer cells and significantly increases cell survival following PpIX-PDT (84). The autophagy activity is dependent on HIF-1α since this transcription factor recognizes a hypoxia response element (HRE) in the promoter of expression of the vacuole membrane protein 1 (VMP1), a protein capable of inducing the formation of autophagosomes (84) (**Figure 2**).

The cancer expression profile of drug-efflux mediators has been involved in multidrug tumor-resistance (MDR) against chemotherapeutics such as imatinib, doxorubicin, and mitoxantrone, as well as PDT (79, 85–91). The ATP binding cassette (ABC) superfamily transporters (e.g. ABCG2 and ABCB1) were found to extrude PS out of the tumor cells (86–88). Despite some mutations on ABCG2 (e.g. R482G, R482T) not affecting PS transport, the Q141K polymorphism may explain increases in the patient photosensitivity to PDT on account of a lower PS efflux (89, 90). On the other hand, the ABCG2 overexpression has been suggested to render the incorporation of some photosensitizers with chemical similarity to pheophorbide A (PhA), including Ce6, MPPa, and 5-ALA (89). Noteworthy, the photosensitizers m-THPP and m-THPC may provide a more effective PDT response even in ABCG2-overexpressing bronchoalveolar carcinoma H1650 MX50 cell line (89). To improve PDT-efficacy considering the ABCB1-mediated PS extrusion, there are several new protocols in development including those on zinc phthalocyanine tetrasulfonic acid and nanotechnology approaches (79, 92). Another way to overcome PS-efflux is to promote a multifunctional drug delivery system (e.g. endocytosis), in which lysosome highlights as a targeted organelle (69), as well as the PDT combination with ABCG2 inhibitor Ko143 (93). Human glioblastoma T98G cells with the highest *ABCG2* expression levels showed relevant synergic death after the PpIX-PDT plus Ko143 in response to increased 5-ALA incorporation (93).

Recently, TPPS-loaded nanogels through its endocytic internalization and pH-sensitive framework might elude photo-oxidation toward multidrug-resistant cancer cells (94). Noteworthy, this approach also remarkably modulates autophagy, whose inhibition may alleviate PFKFB3-elicited tumor dormancy (95). PFKFB3 functions as a regulator of cyclin-dependent kinase 1, linking glucose metabolism to cell proliferation and survival, as well as apoptosis prevention. Depending on the physicochemical PS properties (e.g. pKa), the endo/lysosomal entrapment phenomena may occur during PDT (86), as reported by multiple hydrophobic weak-base drugs (e.g. sunitinib, doxorubicin) (96, 97).

Several strategies have been proposed to overcome PDT-resistance on PS-specificity (70–72, 79). Most of them adjust the PS chemical structure by targeting specific membrane components, which may lessen PS-extrusion. For example, the covalent introduction of a phospholipid to generate porphyrin-lipid derivatives might deal with ABCB1-mediated BPD extrusion and alleviate PDT-resistance of tumor cells (88). It is noteworthy that the 'unconjugated' form of the same porphyrin-lipid does not mitigate the BPD efflux by ABCB1 in breast cancer cells (88). The covalent conjugation of indocyanine green (ICG) and TNYL peptide onto the surface of gold nanospheres (HAuNS) was found to overcome the PDT-resistance (72). Also, Liu et al. have proposed the molecular linkage of the nano photosensitizer to a BCL-2 inhibitor as an adjuvant intervention strategy to increase the PDT efficacy in relapsed-tumor cells (71). Kralova et al. demonstrated that PDT-resistance may be related to PS lipophilicity (86). While glycol porphyrins suffer ABCB1-mediated drug-extrusion, the elicited PDT-resistance associated with the highest lipophilic structure of termoporfin relies on the PS-lysosome sequestration (86).

Also, the protein dysregulation involved in PS-influx must be considered. The 5-ALA influx transporters, such as ABCB6 and SLC15A1/PEPT1, play a pivotal role in the PS-uptake, whose overexpression might increase the PDT efficacy depending on the PS type and the subcellular specificity (91, 98). Thereby, their genetic profiles might determine phototherapy efficacy in dormant cancer cells responsible for disease latency, late metastasis, and tumor relative relapse to chemotherapy and radiation (80, 99).

Aside from less PS accumulation or reduction on ROS generation, the tumor molecular adaptation regarding signaling pathways (e.g. MAPK/JNK/p38α, AMPK, and AKT/mTOR) have also provided PDT-resistance with crosstalk between apoptotic machinery (e.g. BCL-2, BCL-xL, survivin, caspases, and PARP1) and autophagy, as summarized in **Figure 2** (60, 74, 76, 100–103). To overcome ATP depletion due to mitochondrial photo-oxidation, tumor cells activate the canonical energy-sensing AMPK mechanism (104). After phosphorylation AMPK becomes active and leads to Rheb/mTORC1 inhibition with consequent induction of lysosome biogenesis and autophagy, which may dictate the tumor PDT-resilience (102, 104). Also, the acquired tumor resistance to $TPCS_{2a}$-PDT likely occurs due to higher expression of the EGF receptor (i.e., EGFR) and loss of the MAPK/p38 inducing death pathway (76). Indeed, by targeting EGFR the $TPCS_{2a}$-PDT-resistance is significantly reduced regardless of the tumor adaptation respecting the cell death mechanism (e.g. apoptosis, necroptosis, or autophagy), **Figure 2** (76).

The phototoxic PDT-effects might be abrogated by antioxidant defense mechanisms, including ROS-scavenger proteins glutathione, ferrochelatase (FECH), heme oxygenase (HO-1), glutathione peroxidase 4 (GPX4), and glutathione S-transferase Pi 1 (GSTP1) (93, 105). Besides, the heat shock protein 27 (HSP27) may also play a pivotal role in tumor resistance to the mediated-photooxidative stress, e.g. against Photofrin™ (106) or 5-ALAm-PDT, which was related to the

activation of autophagy-based recurrence (107). Paradoxically, its downregulation leads to a relevant decrease in HSP70 expression under hematoporphyrin-PDT, which was associated with an increase in autophagy (108) or apoptosis lessening (109).

Another type of resistance involves nitric oxide (NO) generation through inducible nitric oxide synthase (iNOS/NOS2) in tumor cells. NO has a short life (i.e., <2 s in H_2O) depending on the $^3O_2$ concentration, and is freely diffused as a bioactive free radical interacting with other biomolecules and membranes by hydrophobic partitioning. Besides, NO reversibly impairs mitochondrial respiration through competitive cytochrome oxidase inhibition (110). Several reports revealed that the photooxidative stress may activate the inducible NO synthase isoform (i.e., iNOS), which catalyzes the L-arginine conversion to citrulline and NO in a Ca^{+2}-independent manner and the expense of NADPH and O_2 (111). The PDT-induced iNOS activation may virtually increase NO at the micromolar concentration, which reacts with superoxide ion ($O_2^{\cdot-}$) to give peroxynitrite ($ONOO^-$), a strong oxidant that damages both DNA and unsaturated membrane phospholipids, as reviewed by Tsubone et al. (112). Such NO has been shown to modulate tumor PDT-resistance, which was first demonstrated for Photofrin™ through an *in vivo* preclinical test to treat cancer (113). Subsequently, several reports revealed that the iNOS-derived NO might play a pivotal role in the adaptation and survival of breast (100, 114–117), glioma (103, 118, 119), and prostate (120) cancer cells to 5-ALA-PDT oxidative stress. Autophagy activation may modulate the iNOS expression in response to the suppression of AKT/mTOR signaling *via* ROS generation by UCNPs/Ce6-PDT (121).

5-ALA-PDT also triggers NO-adaptative resistance *via* activation of the PI3K/AKT signaling, leading to NFκB-mediated transcription of iNOS, **Figure 2** (100, 120, 122). Such iNOS upregulation increases NO that modulates cytoprotection against the photo-stress, including apoptosis abrogation, MAPK1/3 deactivation, invasion/migration, and tumor pro-growth (100, 103, 114–120). The adaptative response of tumor cells to PDT-generated oxidative stress (i.e., increased NO) correlates with inhibition of the pro-apoptotic role of MAPK/JNK/p38α pathway (100, 114), with consequent downregulation of the anti-apoptotic proteins survivin, BCL-2, and BCL-xL, lessening the caspase-dependent apoptosis (117, 122). As proposed by *Girotti* the tumor antagonism mediated by the iNOS/NO axis may promote further PDT-resistance pro-growth, invasion, and migration of tumor cells, leading to cancer recurrence (123). To improve 5-ALA-PDT outcome several approaches have been proposed, including iNOS non-specific activity inhibitors (e.g. L-NAME or L-NNA), iNOS specific inhibitors (e.g. 1400W or GW274150), NO scavenger (e.g. cPTIO), NFκB inhibitor (e.g. Bay11) or iNOS-knockdown (100, 103, 114–120).

Another molecular mechanism related to tumor adaptation and resistance against 5-ALA-PDT photo-oxidation relies on the anti-necrotic role of NFκB *via* the increase in AKT/mTOR signaling, at least for glioblastoma U87 and LN18 cells (103). Likewise, 5-ALA-me through photooxidative stress enhances HIF-1α that alleviates cell demise due to an increase in expression of VMP1, which plays a vital role in autophagy initiation (84, 124). On the other hand, 5-ALA-photoinduced stress may activate autophagy *via* AMPK signaling, whose chemical negative regulation results in less caspase-9 activity, and in turn, death suppression, and PDT-resistance (104). Upon ER photoinduced stress autophagy activation contributes to adaptation and rescue of the cellular homeostasis upon RO damage by hypericin-PDT (60). Autophagy abrogation in ATG5-silenced cells increases the PERK/eIF2α/CHOP cascade in response to augmentation of chaperones HSPA5 or GRP78/BiP after hypericin-PDT induced ER-stress (125). It seems that the correlation between the levels of proteotoxicity and the amount of ROS relies on selective autophagy towards the damaged endoplasmic reticulum (i.e., reticulophagy). Therefore, any process that alleviates proteotoxicity and ER-stress might lead to functional consequences, including anticancer immunity (125–127), as well as chemosensitivity (128). For more detailed information see our recent review (112).

Whereas autophagy suppression (i.e., *ATG5* knockdown or 3-MA) increases tumor cells' death, in non-malignant cells (e.g. fibroblasts and murine embryonic fibroblasts, MEFs) autophagy-deficiency paradoxically alleviates mitochondrial cytochrome *c* release, caspase 3 activation, PARP1 cleavage, and turn apoptosis induction. Besides, such ATG5-deficiency leads to clearance of oxidized proteins and reduces photokilling by hypericin-PDT probably through up-regulation of LAMP2A, a receptor for another type of autophagy, i.e., chaperone-mediated autophagy (60). Nevertheless, LAMP2A knockout implicates in apoptotic death correlated with the upregulation of caspase-3 and poly (ADP-ribose) polymerase 1 (PARP1) (60). There is interesting crosstalk between autophagy regulation and PARP1 that may evoke resistance to PDT (101). Besides, cancer stem cells (CSC) are essential players for PDT-resistance and tumor regeneration. Consequently, Wei et al. demonstrated that autophagy in colorectal cancer stem-like cells promotes resistance to PDT-induced apoptosis (129). By isolating PROM1/CD133$^+$ stem-cells they revealed a significant and specific increase in autophagy in response to PpIX-PDT (129). Interestingly, autophagy inhibition and PDT concomitantly elicit higher apoptosis induction, and so, *in vivo* tumorigenicity alleviation (129). Therefore, autophagy plays dichotomic roles in the determination of the cellular resistance or sensitization to PDT-mediated oxidative-stress. This paradigm will further be considered along with the studies discussed in the next section.

PDT-MEDIATED AUTOPHAGY REGULATION IN TUMOR CELLS

PDT triggers autophagy (or macroautophagy) in tumor cells by suppressing the AKT-mTOR signaling (60) or up-regulating the AMPK pathway (102, 104), as summarized in **Figure 3**. The negative effects of the TSC1/2 complex on the mTORC1 activator Rhe may be regulated by AMPK or AKT signaling (**Figure 2**). Also, autophagic machinery may be transcriptionally

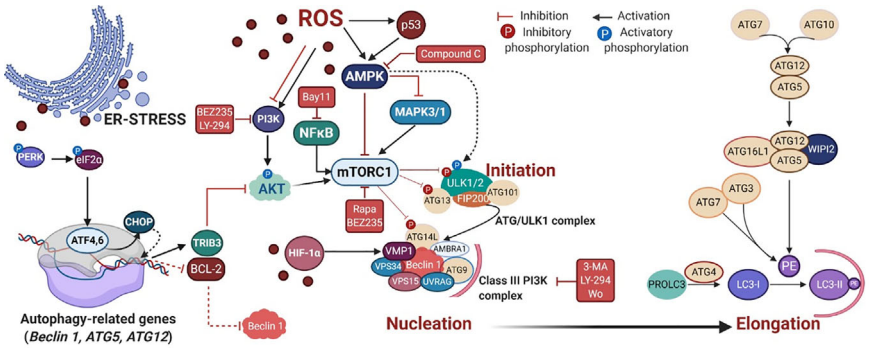


FIGURE 3 | PDT-mediated autophagy regulation in mammalian cells. The reactive oxygen species (ROS)-mediated mechanism covers the autophagy regulation *via* several signaling cascades, including the energy-sensing AMPK and the PI3K/AKT/mTOR pathways. Following mTOR inhibition, the autophagy initiation starts through the activation of the ATG/ULK1 complex that translocates to the ER membrane and recruits ATG9, providing membrane components to the phagophore (130, 131). ULK1 complex can also be alternatively activated by AMPK (132, 133). In the nucleation process, following activation of the Class III PI3K complex I, occurs the production of phospholipid phosphatidylinositol3-phosphate (PI_3P) which recruits PI_3P-binding proteins (e.g. WIPI2), resulting in the change of the ER membrane structure with its elongation to form a phagophore (134). The elongation step relies on the generation of the soluble cytosolic LC3-I that becomes LC3-II after conjugation to the head group of the lipid phosphatidylethanolamine (PE), which occurs through a cascade of ubiquitin-like reactions involving ATG enzymes (e.g. ATG7, ATG3, and ATG5-ATG12-ATG16L). Next, LC3-II is attached to the lumenal and cytosolic surfaces of autophagosomes. The drug mediators that activate (BEZ235, rapa, and LY-294) or inhibit (3-MA, compound C and wo) autophagy machinery are depicted in red. Rapa = rapamycin, LY-294 = LY-294002, wo = wortmannin. Picture created with BioRender.com.

regulated (e.g. ATF4, ATF6, CHOP, and p53) in response to cytoplasmic or organelle photo-oxidation (128, 135, 136). Photo-oxidation enhances HIF-1α/VIMP1-mediated autophagy induction (84, 124). There are other pathways triggered by PDT capable of regulating autophagy machinery, e.g. NFκB (103) and MAPK1/3 (74) (**Figure 3**).

Depending on the extent of mitochondrial photodamage, tumor cells elicit mitophagy to rescue cellular homeostasis through clearance of oxidized or depolarized mitochondria. Mitophagy has several distinct variants (*i.e.*, type 1, 2, and 3) and prevents the release of proapoptotic proteins, generation of toxic mitochondrial-derived ROS, and futile ATP hydrolysis (137–140). A primary cellular response following the mitochondrial photodamage is the recruitment of the E3 ubiquitin ligase PRKN/parkin to the mitochondrial outer membrane, which depends on PINK1 (59, 141). Once recruited, PRKN ubiquitinates several outer membrane proteins marking mitochondria for 2 mitophagy (140, 142).

The cellular responses against the photo-stress also involve selective autophagy known as reticulophagy that removes oxidized ER subdomains. Despite not yet being fully understood, there are two main ER-resident proteins prone to interact with LC3-II, i.e., reticulophagy regulator 1 (RETREG1/FAM134B) and cell cycle progression 1 (CCPG1) (143). During the PDT-mediated reticulophagy, ATF4 or CHOP upregulates the expression of TRIB3 or autophagy-relevant proteins (ATG5,

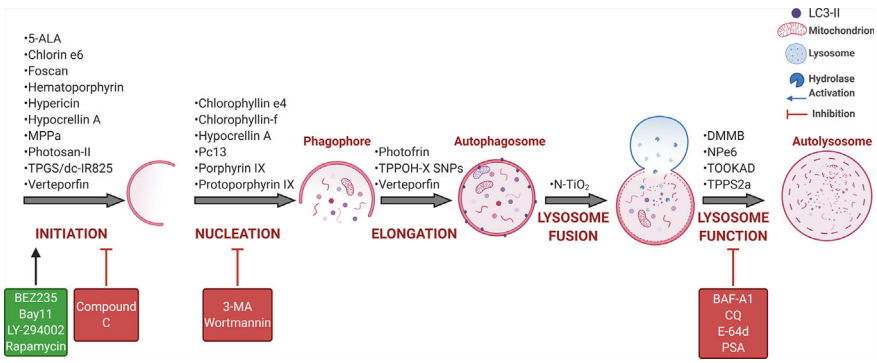


FIGURE 4 | PDT-mediated autophagy regulation in tumor cells. Several PSs were described as phototherapeutic modulators of autophagy flux, which may be further activated (green) or inhibited (red) by some drugs, including the Class I PI3K/mTOR inhibitor (BEZ235), NFκB inhibitor (bay11), pan-the Class I PI3K inhibitor (LY-294002), or mTORC1 inhibitor (rapamycin), AMPK inhibitor (compound C), Class III PI3K/VPS34 inhibitor (3-MA or wortmannin) and lysosome inhibitor (BAF-A1, CQ, E-64d, or PSA) (104, 150–156). BAF-A1 also blocks the lysosome fusion with autophagosomes that occurs independently on intralysosomal pH and relates to reduced ATP2A/SERCA activity (152, 157). For more details, see **Tables 1** and **2**. Art was created with BioRender.com.

ATG12, and Beclin 1), as well as downregulates the expression of BCL-2 (62, 135, 136). The PDT-mediated autophagy can be chemically regulated by some drugs as depicted in **Figure 3**, such as rapamycin, BEZ235, LY-294000, Compound C, 3-MA, and wortmannin (56, 60, 100, 103, 104, 144–147).

PDT of early response genes by the hyperactivation of the survival pathway, resulting in overexpression of anti-apoptotic (BCL-2, survivin, BCL-xL) or autophagy-related proteins, evoking PDT-resistance (68, 122). Recently, the tumor resistance to several antitumor agents (e.g. cisplatin, oxaliplatin, carboplatin, doxorubicin, etoposide, rapamycin, everolimus, alpelisib, pictilisib, and AZD8055) was related to elevated and sustained activation of the PI3K/mTOR signaling pathway (148). Curiously, in these PI3K/mTOR-activated cells, the promotion of energy metabolism stress (e.g. 2-DG/DCA) led to apoptosis due to the sustained blockage of the pro-survival autophagy (148). Hence, photo-damaging organelles, such as mitochondria, lysosomes, or reticulum endoplasmic seem to be amenable to mediate death in drug-unresponsive tumors.

Evidence relating to the important role of autophagy in the PDT context continues to accrue (63). Owing to the high reactivity of photogenerated ROS, selective autophagy is initiated to remove oxidatively damaged organelles, such as mitochondria (i.e., mitophagy), lysosomes (i.e., lysophagy), endoplasmic reticulum (i.e., reticulophagy), and, or peroxisomes (i.e., pexophagy), which are intracellular targets of

TABLE 1 | PDT-mediated autophagy: cytoprotective role and modulatory guidelines to increase tumor outcome.

PS	Target	Experimental model	Autophagy activation	Therapeutic modulation of the autophagy machinery		Outcome	Ref.
				Chemical	Genetic		
5-ALA	Mito	Human glioblastoma (U87 and LN18) and breast cancer (COH-BR1)	PI3K/AKT/mTOR	BAF-A1 Wortmannin Bay11	ATG7[A]	↑apoptosis, ↑necrosis ↑LDH	(100, 103)
Chlorophyllin e4	Lyso	Human bladder cancer (5637 and T24)	Beclin/LC3-II increase	3-MA BAF-A1		↑apoptosis	(55)
Chlorophyllin f	Lyso Mito	Human bladder cancer (5637 and T24)	LC3-II increase	3-MA		↑apoptosis	(54)
Hematoporphyrin	Memb.	Human oral cancer (Fadu)	AKT/mTOR	3-MA		No effect	(101)
Hypericin	ER	Human cervix carcinoma (HeLa)	AKT/mTOR	3-MA	ATG5[A]	↑apoptosis ↑cleaved PARP1 ↑caspase 3	(60)
Hypericin low dose (<100 nM)	ER	Human oxaliplatin-resistant colon cancer (HCT116, HCT8) and HCT116/L-OHP murine model	↑GRP78/↓LC3II/I ↓SQSTM1	3-MA 4-PBA	Beclin 1[A] CHOP[A]	↑apoptosis ↓out-growth ↓Ki67 ↑oxaliplatin sensitization	(128)
Hypocrellin A	Mito	Human cutaneous squamous carcinoma (cSCC)	JNK/NFκB pathway	3-MA Bay11		↑apoptosis ↓BCL2 ↑caspase 3 ↑BAX	(146)
Pc13	Mito	Human melanoma (A375)	BCL-2/LC3-II increases	CQ 3-MA Wortmannin		↑apoptosis ↑cleaved PARP1	(74)
Photofrin™	Mito	Human cervix carcinoma (HeLa) and breast cancer (MCF7)	LC3-II increase	3-MA BAF-A1	ATG5[B] ATG5[C]	↑apoptosis ↑cleaved PARP1 ↑caspase-3	(161, 162)
Photosan-II	Mito	Human colorectal cancer (HCT116 and SW620), and SW620 derived xenografts	ATK/mTOR and AMPK pathway	CQ	ATG7[A]	↑apoptosis ↑cleaved PARP1 ↑LC3-II ↓Tumor mass	(102)
Porphyrin IX	Mito	Human colon cancer (HCT116)	BCL-2/Beclin 1/ ATG7/LC3-II	CQ	ATG7[A]	↑apoptosis ↑caspase 3 ↑LC3-II ↑SQSTM1	(163)
Protoporphyrin IX	Mito	Human colon cancer (HT29 and PCC)	Beclin 1/ATG7/ ATG5-12/LC3-II	3-MA CQ	ATG3[B] ATG5[B]	↑apoptosis ↑caspase 3	(129)
TPPOH-X SNPs	Lyso	Human colon cancer (HT29)	LC3-II increase	3-MA		↑apoptosis ↑caspase 3 ↓LC3-II	(164)
Verteporfin	Mito	Murine hepatoma cells (1c1c7)	PI3K/AKT/mTOR	CQ	ATG7[B]	↑apoptosis	(165)

Lyso, lysosome; memb, intracellular membranes; mito, mitochondria; ER, endoplasmic reticulum; 3-MA and wortmannin, Class III PI3K inhibitors; Bay11, NFκB inhibitor; CQ and BAF-A1, inhibitors of lysosome function; [A]: siRNA, [B]:shRNA; [C]: CRISPR/Cas9.

TABLE 2 | PDT-mediated autophagy: death routine and modulatory guidelines.

PS	Target	Experimental model	Molecular mechanism	Autophagy flux and death routine	Autophagy modulation	Outcome	Ref.
5-ALA	Mito	Human lung cancer (CL10 and PC12)	AMPK/MAPK/mTOR	Boosting: AMCD?	3-MA	↑survival ↓caspase-9 and 3	(104)
Verteporfin	Mito	Human prostate cancer (PC3)	Inhibition of autophagosome formation	Dysfunctional: AACD	BEZ235 LY-294002	↑apoptosis ↓cleaved PARP1 ↑LC3-II	(144) (145)
Ce6	Mito	Human breast cancer (MCF7 and MCF7/ADR)	?	?	3-MA BAF-A1	↑survival	(166)
		Human breast cancer (MDA-MB-231 and MCF7)	AMPK	Boosting: AACD	3-MA + 2-DG	↑apoptosis ↑pAMPK ↓Beclin 1 ↓LC3-II ↓Tumor mass	(167)
DMMB	Mito Lyso	Human cervix carcinoma (HeLa), hepatocarcinoma (HepG2), and melanoma (SKMEL18 and 25)	Mitophagy/Lysosomal dysfunction	Dysfunctional: AACD ↑PRKN, ↑SQSTM1, ↑LC3II, ↑autolysosomes accumulation	3-MA BAF-A1	↓survival Non-changed	(59)
Hypericin high dose (500 nM)	ER	Human oxaliplatin-resistant colon cancer (HCT116, HCT8) and HCT116/L-OHP murine model	GRP78/CHOP/AKT	Boosting: AACD ↓SQSTM1, ↑LC3II/I	3-MA ATG5* Beclin 1*	↑out-growth ↑Ki67 ↓oxaliplatin sensitization	(128)
MPPa	ER	Human osteosarcoma (MG-63)	JNK pathway	Boosting: AMCD? ↓BCL-2, ↑ Beclin 1, ↑LC3II/I, ↓SQSTM1	3-MA CQ	↓apoptosis ↓LC3-II ↓caspase 3	(168)
		Human breast cancer MDA- MB-23 and murine model	PERK/eIF2α/CHOP	Boosting: AACD ↑ Beclin 1, ↑LC3BII/I, ↓SQSTM1	3-MA	↓apoptosis ↑out-growth ↓LC3B-II	(62)
		Human osteosarcoma (MG-63)	PERK/IRE1α/CHOP CHOP/AKT/mTOR	Boosting: AMCD? ↑LC3II/I,↓SQSTM1	Rapamycin	↑apoptosis ↓pMTOR ↑LC3-II ↓SQSTM1 ↑caspase 3 ↑ cleaved PARP1	(147)
mTHPC Foscan®	ER	Human breast (MCF7), lung (A-427), oral cavity (BHY), esophagus (KYSE-70), bladder (RT-4), and cervix (SISO) cancer	GRP78/LC3-II	Boosting: AMCD? ↑LC3II/I	Wortmannin PSA E-64d	↓apoptosis ↓LC3-II ↓caspase-9 ↓caspase-3 ↑LC3-II	(56) (155)
NPe6	Lyso	Murine hepatoma (1c1c7)	Lysosomal dysfunction	Dysfunctional: AACD? ↑LC3II/I, ↑vacuolization	ATG7*	↑survival ↓caspase activation	(169)
N-TiO_2 nanoparticles	Lyso	Human melanoma (A375)	Impairment of lysosomal fusion with autophagosome	Dysfunctional: AACD ↑LC3II/I, ↑SQSTM1 ↑RIPK1, ↑HMGB1	3-MA BAF-A1 Necrostatin-1	↓necroptosis ↑cellular rescue (90%)	(170)
TPPS2a	Lyso	Human cervix carcinoma (HeLa)	Lysosomal dysfunction	Dysfunctional: AACD ↑LC3II/I, ↑vacuolization	3-MA	Slightly decrease	(35)
TPGS/dc-IR825	Mito	Human lung cancer (A549) and xenografts	Mitophagy/AMPK	Boosting: AACD ↑PINK1, ↑PRKN, ↑LC3II/I, ↑SQSTM1, ↓ATP	CQ 3-MA	↑survival ↓PINK1 ↓pAMPK	(141)
WST11 TOOKAD®	Lyso	Murine hepatoma (1c1c7)	Lysosomal dysfunction	Dysfunctional: AACD? ↑LC3II/I, ↑vacuolization	ATG5* ATG7*	↑survival ↓caspase activation	(169)

*Lyso, lysosome; mito, mitochondria; ER, endoplasmic reticulum; 3-MA, LY-294002, and wortmannin, Class I/III PI3K inhibitors; BEZ235, Class I PI3K/mTOR inhibitor; rapamycin, mTORC1 inhibitor; BAF-A1, CQ, E-64e, PES, inhibitors of lysosome function; * genetic silencing/knockout.*

several photosensitizers (149). Despite controversial findings concerning autophagy activation *via* ROS generation following PDT, there is now a consensus about the underlying mechanisms regarding cytoprotection or death.

Over the past ten years, some questions have been addressed by several authors using cancer cell lines and distinct PDT protocols. As summarized in **Figure 4**, PDT protocols with different PSs were found to therapeutically modulate autophagy.

Note also that the complexity of autophagy and numerous steps allows for several possibilities of intervention, but the performance in PDT does not come to simple conclusions, i.e., a better understanding of its role is still necessary. To avoid controversial analysis of the real role of autophagy (i.e., cytoprotective *versus* death routine), the autophagy community appeals to a straightforward effort in following robust guidelines to monitor autophagy (158–160). As summarized in **Tables 1** and **2**, numerous *in vitro* or *in vivo* studies have been conducted to describe the autophagic pivotal role in PDT. Herein, we will briefly discuss this paradigm.

The Pro-Survival Autophagy Role

In general, the therapeutic effects of PDT are related to apoptosis or necrosis, and autophagy might be a double-edged sword, depending on the type of photosensitizers and cells (19). Instead of promoting cell death *per si*, autophagy often accompanies the cellular demise by PDT, as a last attempt of cells to cope with oxidative stress and to survive. Several *in vitro* and *in vivo* reports have demonstrated considerable evidence that autophagy plays a pivotal cytoprotective role that virtually occurs along with other RCD, like apoptosis, necroptosis, necrosis, or parthanatos (54, 55, 60, 74, 100–103, 128, 129, 146, 161–165, 171–174). The description of such cell death mechanisms will not be considered herein.

Autophagy is a key point on survival and tumor adaptation, whose inhibition decreases anti-apoptotic proteins' expression (e.g. BCL-2 and survivin) or increases pro-apoptotic proteins, such as BAX, leading to tumor sensitization to photo-stress, e.g. 5-ALA-PDT (68). However, this protective response may be compromised *via* photooxidative-mediated NFκB activation through induction of an adaptative AKT/mTOR/S6K response that leads to the alleviation of necrotic cell death (103). The apoptotic machinery (e.g. upregulation of cytochrome *c* release, BAX, caspase-3, and PARP1) was found to occur accompanying the protective autophagic signals in response to Photosan II-PDT *via* activation of the AMPK pathway or suppression of the AKT/mTOR signaling (102). Notably, the protective autophagy is responsible for cell adaptation and delay of PARP1-mediated apoptosis at low dose hematoporphyrin-PDT (101).

Autophagy was found to protect photosensitized cells from oxidative damage triggered by several photosensitizers, like 5-ALA (100, 103), chlorophyllin e4 (55), chlorophyllin-f (54), hypericin (60, 128), hypocrellin A (146), Pc13 (74), Photofrin™ (161, 162), protoporphyrin IX (129), and porphyrin IX (163), TPPOH-X SNPs (164), and verteporfin (165). This cytoprotective autophagy can be alleviated trough chemical (e.g. BAF-A1, CQ, 3-MA, or wortmannin) and genetic inhibition of essential autophagy-related genes (e.g. *ATG3*, *ATG5*, *ATG7*, or *Beclin 1*) or autophagy regulators (e.g. *CHOP*), leading to significant suppression of PDT-resistance of tumor cells (**Table 1**).

Thereby, photoinduced cellular stress could be targeted to further death through negative modulation of autophagy. Xiong et al. demonstrated a promisor therapeutic association-targeting autophagy to overcome PDT-resistance of colon cancer xenografts (102). Protoporphyrin IX-PDT in colorectal cancer stem-like cells (CCSCs) failed to initiate out-growth in almost 70% when associated with autophagy inhibitors (e.g. CQ and *ATG5* shRNA), compared to 25% in PDT alone. Thus, autophagy inhibition can be considered as a target to deal with adaptation or resistance to photooxidative stress, leading to higher antitumorigenicity of PDT in tumor-xenografts (102, 129).

Whereas most of the PDT-protocols trigger autophagy as cytoprotective, fewer propose to activate autophagy as a death routine in cells succumbing to photooxidative stress, as summarized in **Table 1**. By analyzing reliable experimental studies, we propose in the next section to cope with the challenge to distinct autophagy-associated death modes, considering the high variability of cellular responses and different types of PDT protocols.

The Pro-Death Autophagy Role

In general, photoinduced cell death in the mammalian cells is preceded or accompanied by autophagic vacuolization, a morphological alteration that may be considered as an example of the widespread belief of a "*type II programmed cell death*" or "*autophagic cell death*" (175). However, both terms are unappropriated following recent guidelines (160). On July 15, 2020, a Medline search of "*autophagic cell death*" or "*autophagic death*" and "*PDT*" or "*Photodynamic Therapy*" yielded 17 entries, which constitutes a fraction - close to 10% - of all 184 articles published on the topic "*autophagy*" in the PDT field. This led us to reflect on the expression "*autophagic cell death*" (57) after photodamage. Autophagy can protect cells and help them to tolerate the photodamage (60, 129); however, if there is a high level of autophagy or blockade flux, "*autophagic cell death*" could probably occur. Herein, we review this paradigm and – polemically – raise doubts about the existence of "*autophagic cell death*" mediated by PDT.

Incontestably, converting a protective autophagic mechanism to a destructive or lethal avenue is now being well-defined as autophagy-dependent cell death (ADCD) as postulated by the international committee on cell death (176). Using ADCD term one would postulate that the photoinduced death is autophagy-regulated through its machinery and thereof components, whereas its pharmacologic or genetic lessening would lead to less death regardless of other RCD mechanisms. Even though most of the reports have evaluated increased autophagic flux and puncta vacuoles, none of them formally establishes autophagy itself (or ADCD) as responsible for photo-induced cell death. Consequently, before ascribing a direct death role to autophagy, it is recommended to determine the machinery efficiency status by generally inhibiting the autophagy pathway using genetic approaches (knockdown or knockout based in siRNA, shRNA, or CRISPR/Cas9), see **Table 2**.

Some reports have shown that depending on the stress level, the autophagic apparatus might intrinsically contribute to other cell death programs, like apoptosis or necroptosis (35, 56, 59, 62, 104, 128, 141, 144, 145, 147, 155, 166–170), see **Table 2**. In spite of not inducing cell death *per si*, the RCD routine autophagy-mediated cell death (AMCD) may be significantly rescued by chemical autophagic inhibition (e.g. CQ, 3-MA, or BAF-A1)

and/or genetic manipulation (e.g. ATG7, Beclin 1), as reported for some drugs (e.g. QW24, PTC-209, or sodium butyrate) (177–179).

During photooxidative damage autophagic machinery seems to play a key role regarding the increase in death mediators, probably leading to AMCD together with apoptosis (56, 147, 168). Another RCD routine linked to autophagy has been proposed, i.e., autophagy-associated cell death (AACD, which may or not occur alongside other cell death modalities, like apoptosis (**Table 2**). AACD commonly relates to the impairment of the early (144, 145) or the late stages of the autophagy flux (35, 59, 169, 170). Based on recent evidence, we propose that the terms "*autophagic cell death*" or "*autophagy death*" should be substituted to "AMCD" or "AACD". However, one should initially consider the main differences between AMCD and AACD in tumor cells succumbing to photooxidative stress. Unlike AACD (35, 59, 62, 128, 141, 167, 170), AMCD may require the autophagic machinery to intrinsically regulate apoptosis (56, 104, 147, 155, 168), as summarized in **Table 2**.

The engagement of autophagy as a death route does not occur naturally, instead, quite specific experimental conditions should be followed, including the PDT exposure dose, type of protocol sub-sequentially or parallel photodamage on intracellular targets), type of targeted organelle (e.g. lysosomes or ER), availability of cellular machinery to evade apoptosis. Whereas in the case of cytoprotective autophagy the pharmacologic or genetic autophagic lessening sensitizes tumor to higher PDT-photoinduced death (**Table 1**), in the case of pro-death autophagy may occur either no effect or substantial alleviation of the PDT-photokilling (**Table 2**). Nevertheless, autophagy as a death routine remains allusive and still requires more studies.

The autophagy-related death can be either related to AMCD or AACD, depending on the dose, physicochemical properties, and the intracellular specificity. Lange et al. showed different autophagy responses concerning the Foscan®-PDT doses (e.g. LD_{50} versus LD_{90}) (155). While LD_{50} dose leads to moderate ER-stress with less apoptotic cell death and probable autophagic response, high-dose PDT (i.e., LD_{90}) by damaging proteins involved in the autophagic machinery triggers pro-death autophagy associated with activation of apoptotic hallmarks, such as cleaved PARP1, phosphatidylserine membrane externalization (155). A similar regulation was described in mutated caspase-3 breast cancer cells (e.g. MCF7) (56). While autophagosome formation accompanies cleavage of pro-caspase 7 and PARP1 at the LD_{90} dose´s Foscan®-PDT, chemical inhibition of the autophagy flux lessen the pro-death autophagy, leading to a decrease in procaspase activation and less cytotoxicity (56). The MPPa-PDT also may activate pro-death autophagy *via* a ROS-dependent JNK/Beclin 1 pathway, which intrinsically enhances procaspase-3 activation (168). By suppressing the early (e.g. 3-MA) or late stages of the autophagic process (e.g. CQ), tumor recurrence may increase by up to 70% (168).

Recent reports revealed that MPPa- ER photo-stress could intrinsically regulate the pro-death autophagy *via* a PERK/CHOP/AKT/mTOR signaling, with consequent boosting autophagy flux and activation of PARP1, procaspase 3 and 12 (62, 147). Probably, as a secondary response of mTOR on the phosphorylation of S6K or 4EBP1, the ROS-mediated effect on the PERK pathway leads also to cell arrest, decrease in invasion and migration, due to respectively, downregulation on cyclins (A, E and B1) and metalloproteinases (MMP-2 and -9) (147). Such MPPa-triggered downregulation of MMP-2 and -9 was due to the ROS-mediated inhibition of AKT/NFκB/mTOR signaling with suppression of the metastatic behavior of breast MCF-7 cancer cells in xenografts (180). Chen et al. demonstrated that rapamycin (mTOR inhibitor) enhances the phototoxicity related to MPPa-mediated pro-death autophagy with a consequent decrease in SQSTM1/P62 levels and increased cleavage of procaspase-3 and PARP1 (147). Even though this pro-death autophagy role should be more investigated regarding the elicitation of the autophagic machinery to cleavage PARP1 and procaspases, these findings are suggestive of an AMCD routine.

Lin et al. revealed that the correlation between AMCD and ER photo-stress occurs *via* CHOP following hypericin-PDT at high doses (128). They proposed that pro-death autophagy occurs in the case of high ER photo-stress, which may relieve chemo-resistance towards oxaliplatin (128). Paradoxically, a low level of ER photo-stress mediated by hypericin-PDT leads to the opposite role of the autophagy process (i.e., pro-survival), *via* downregulation of AKT/mTOR signaling in HeLa tumor cells, which enhances PDT-mediated death by 50% after negative modulation of autophagy (e.g. *ATG5* siRNA or 3-MA) (60). Thereby, depending on the level of damage (low or high dose) mediated by hypericin-PDT, autophagy may contribute distinctively in apoptosis-resistant tumor cells (128).

The type of activation mechanism (e.g. AMPK) regardless of the type of PS (i.e., 5-ALA or Ce6) also leads to pro-death autophagy (104, 167). Although this interpretation is correct, it is still not possible to correlate pro-death autophagy with PS type and subcellular localization. PDT activates the pro-apoptotic MAPK/JNK/p38α pathway (100, 114) but also negatively regulates the AMPK phosphorylation (104). Parallel to AMPK activation, there is a decrease in the caspase-3 activity, an increase in the ATP depletion, and deactivation of the MAPK1/3 pathway (104). That MAPK deactivation would lead to mTOR activation, with consequent inhibition of autophagy, is contrary to the sustained AMPK activation that maintains elevated as an adaptative response to 5-ALA-PDT (104). Consequently, AMPK seems to be a key point in the PDT-response and autophagy activation. Indeed, through AMPK activity abrogation by compound C or VPS34 inhibition (3-MA mediated) the cell survival might be partially rescued, possibly due to the autophagy-independent mitochondrial photodamage (104). Corroborating this finding, Ce6-PDT also activates AMPK that is further enhanced upon glycolysis inhibition by 2-DG, with consequent tumor regression *in vitro* and *in vivo* (167). The AMPK hyperactivation relates to high ATP depletion, which leads to an increase in Beclin 1 and LC3 lipidation that could be lessened by 3-MA (167). Especially in caspase-3 mutated cancer cells resistant to either multidrug (e.g. doxorubicin, cisplatin, and paclitaxel) or

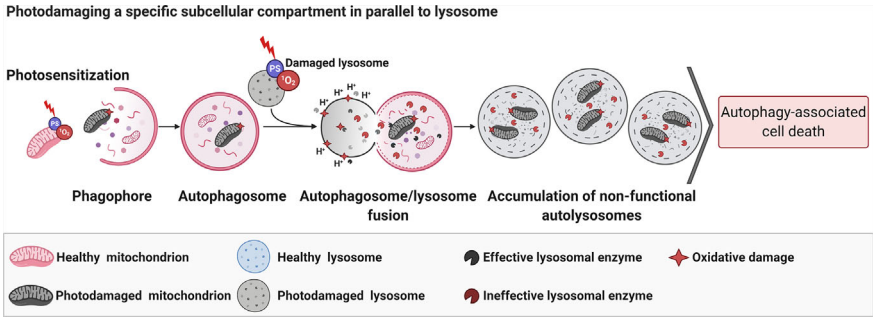


FIGURE 5 | Parallel photodamage in mitochondria and lysosome evolves to autophagy-associated cell death. The PDT-mediated photodamage in mitochondria and lysosomes *per si* leads to efficient autophagy-associated cell death regardless of the chemical modulation of autophagy flux (i.e., BAF-A1 or 3-MA) (59). Figure created with BioRender.com.

Ce6-PDT when the autophagic flux is chemically inhibited (e.g. 3-MA or BAF-A1), the tumor relapse increases up to 50% (166). It seems that the hyperactivation of the AMPK pathway relates to boosted autophagy triggered by photooxidative damage on mitochondria (**Figure 3**). However, it is important to emphasize that the activation mechanisms of AACD remain elusive and poorly understood.

Overall, excessive, or impaired mitophagy/autophagy appears to trigger the AACD routine. The boosted mitophagy activation mediated by TPGS/dc-IR825-PDT exceeds the degradative function of autolysosomes, ending up with huge vacuolization and degradation impairment, as well as depletion of ATP, activation of AMPK pathway, and bioenergetic catastrophe (141). Such effects were also observed in xenografts models when TPGS/dc-IR825 nanomicelles were intravenously injected into tumor-bearing mice showing high tumor accumulation and retention (141). TPGS/dc-IR825 nanomicelles showed a remarkable *in vivo* therapeutic efficiency leading to total tumor remission, possibly related to their minimized cellular-extrusion (141).

When photodamage reduces the number of functional lysosomes and promotes their total disruption (59), or avoids their fusion with autophagosomes (170), AACD is activated. If the lysosomes are slightly photodamaged, only enough to enable an autophagic pro-survival response (probably due to lysophagy), there is a restoration of homeostasis (59). The pro-survival autophagy triggered by PDT (PS at low doses) can be promptly switched to AACD when parallel mitochondrial membrane damage occurs (59). In line with this notion, lysosomes have been considered as promisor targeted-organelle to PDT, even much more when parallel damage in the mitochondria membrane is mediated (59). Some reports corroborated with this premise (181, 182). Following this concept, the PDT-triggered mitophagy activation would fail in the context of lysosomal impairment, which evolves to AACD (**Figure 5**) (59).

Another path to trigger AACD was recently reported (170). Whereas N-TiO_2-PDT induces efficient autophagic flux in the dark condition, its photo-activation compromises pro-survival autophagy. The replacement of the cytoprotective response mediated by photooxidative stress relates to the impairment of the lysosomal fusion with autophagosomes (170). Consequently, there was an increase in ROS production with consequent elicitation of RIPK1/HMGB1-related necroptosis, which is abrogated upon treatment with necrostatin-1, a specific inhibitor (170).

The PDT-triggered molecular mechanisms differ concerning the pro-death autophagy routine, i.e., AMCD or AACD, and the mechanistic framework must be carefully considered before choosing the type of autophagy modulation (e.g. activation or inhibition). For instance, even targeting the same organelle (i.e., lysosomes), the AACD elicitation might differ upon the 3-MA inhibition in the early autophagy. Whereas the photoinduced lysosomal dysfunction promoted by DMMB evokes *per si* tumor death regardless of 3-MA (59), the impaired lysosome/autophagosome fusion mediated by N-TiO_2-PDT leads to a high level of tumor relapse (90%) (59). Thereby, to efficiently relieve tumor recurrence, a better choice should be the lysosomal inhibitor BAF-A1, which slightly increases the N-TiO_2 phototoxicity (170). Meanwhile, when PDT increases AMCD, the boosting autophagy triggered through mTOR inhibition (e.g. rapamycin) should be the best direction to deal with MPPa-PDT-resistance (147). Moreover, the secondary effects regarding mTOR suppression would lessen any invasive or migratory activity of tumor cells (147).

Although autophagy plays a protective role in murine tumor cells photosensitized with lower concentrations of verteporfin (165), at higher concentrations it switches pro-survival autophagy to AACD probably by the inhibition of autophagosome formation in human prostate cancer cells (144). Thereby, in high-phototoxicity doses that compromise autophagy flux instead of inhibiting autophagy it is preferable to modulate its activation through treatment with dual Class I PI3K/mTOR inhibitor, e.g. BEZ235 (144), or pan-Class I PI3K inhibitor, e.g. LY-294002 (145). BEZ235 markedly increased growth inhibition of PI3K mutated-cancer cells (183). These findings highlight that verteporfin-PDT is an independent cancer treatment strategy, capable of overcoming pro-autophagy to deal

with cancer resistance (e.g. against chemotherapy and radiation). However, further *in vivo* studies are still urgent to determine whether such a combination will lessen tumor out-growth.

Based on these pieces of evidence, we can conclude that the efficiency and rate of engagement in causing death after PDT depend on the cell type, the photosensitizer type, protocol details (concentration, light dose, targeted-organelle, and others). As revealed by pre-clinical studies, both AACD and AMCD can be chemically or genetically modulated to increase PDT outcomes, and therefore both mechanisms should be considered as promisor ways to deal with clinical tumor recurrence.

THE IMPROVEMENT IN THE CLINICAL OUTCOME OF CANCER PATIENTS MEDIATED BY PDT

Aside from the PDT-mediated photodamage that intrinsically correlates with regulated cell death, PDT also plays antitumor immunological activity, involving activation of CD^{+4} and CD^{+8} helper T lymphocytes, endothelial damage, the release of inflammatory mediators and cytokines (184–188). Thereby, along with the engagement of cell death PDT outcomes, tumoral remission is also due to its modulatory role in the immune response (187), which could control the disease´s progression to distant sites. Several preclinical pieces of evidence pointed out the promising role of PDT as a therapeutic strategy for tumor local or distant remission supporting; the oncology community also moved forward in the clinical field. Consequently, several clinical trials have been conducted or are in progress. According to *Clinical Trials.Gov*, 186 intervention studies have been carried out so far (189).

Despite all favorable oncological applications of PDT, it still raises urgent debate in medical practice. We shall, therefore, summarize the key issues concerning clinical outcomes, tolerability, and efficacy of PDT using e.g. 5-ALA, HAL, Photofrin™, Foscan®. We have considered only completed or terminated clinical trials, which enrolled at least three patients, most of them had the involvement of apoptosis or necrosis, and we will argue how the regulation of autophagy could improve clinical outcomes.

Basal cell carcinoma (BCC) continues to have increased incidence rates worldwide, especially in Australia where there has been a 4.4-fold increase (190). According to a network meta-analysis of non-melanoma skin cancer treatment, the surgical excision has been considered as the optimal approach with high efficacy, considering the complete response and complete lesion clearance, with moderate adverse effects (191). However, the risk of developing a subsequent lesion in three years after the first one is elevated ranges from 33% to 70%, which probably evolves from compromised histological margins (192). Indeed, even after early surgery intervention, tumor recurrence was observed in 50% of the BCC patients (~7 months after first intervention) (193). In addition to the poor or unacceptable long-term cosmetic outcomes, wide surgical excisions might sometimes require surgical reconstruction (194).

Contemplating the preserved cosmetic effect and safety for treating non-melanoma skin cancer, PDT is highlighted as an alternative strategy to treat BCC (195, 196). According to the randomized phase 3 trial (NCT02144077), the PDT protocol employing the 5-ALA formulated through a non-emulsion gel BF-200, promoted an effective remission response (93.4%) in BCC lesions, with a low cancer recurrence of 8.4%, after a one-year follow-up (197). The trial showed side effects with mild to moderate intensity regarding tolerability and safety, including pain at the treatment site. Another clinical trial corroborates the favorable outcome of PDT for BCC treatment. In a non-randomized phase 1 trial (NCT00985829) with the enrolment of 28 participants, 5-ALA-PDT lead to complete (32%) or partial (50%) remission, without considerable cosmetic impairment, having only low cases of local pain (7.1%). Even though 5-ALA-PDT is a promising therapeutic avenue to tackle BCC, some details of the protocol were not considered or even described, including exposure time and thickness of the photosensitizer applied to the skin, the specific wavelength of the light used, and the clinical outcome of BCC concerning its histologic subtype, e.g. pigmentary, superficial or nodular. Therefore, it is difficult to analyze the reasons for partial remission response (<50%) (NCT00985829). Proper establishment/definition of the dosimetry parameters could improve this result (197). Considering tumor-adaptative response related to sustained AMPK signaling (104), drug-efflux (89) or iNOS/NO axis (123), the combination of 5-ALA-PDT with positive modulators of the autophagic machinery (e.g. rapamycin), regulators of iNOS and drug-extrusion, NO scavengers, or NFκB inhibitors should be considered to increase clinical outcomes (93, 100, 103, 114–120, 147).

In general, the therapeutic approach to tackle head and neck tumors is difficult, considering that recurrence or even the remaining disease may occur in over 40% of the treated patients (198). This scenery maybe even more dramatic in severe cases, including those related to surgeries following neo-adjuvant treatment, or even, in the case of adjuvant radiotherapy. To overcome such difficulties related to internal tumors, the PDT protocol was improved based on the facility to percutaneously deliver light using multiple laser fibers, which are inserted directly into head and neck tumors, named Interstitial photodynamic therapy (iPDT). A phase 1-2 study attempted to assess the efficacy of iPDT using Foscan® as a photosensitizer. This strategy was considered as an alternative rescue therapy to treat recurrent head and neck tumors before surgery, radiotherapy, or chemotherapy (199). After 1 month of follow-up, 20% of the 45 patients treated obtained a complete response (e.g. free disease), whereas half (53%) experienced symptomatic relief (bleeding, pain, or decreased tumor volume). Among those patients with complete response, 33% died due to recurrence disease within an interval of 17 to 32 months. Meanwhile, 56% survived during follow-up time (10-60 months). Notably, 73% of patients survived for at least 16 months. Adverse events such as pain and edema for 2-4 weeks was reported (199). To improve clinical outcomes, the combination of Foscan®-PDT with late inhibitors of autophagy flux might be considered in future studies, which beyond increases to AACD may improve

antitumor immunological activity. This combined approach may increase the proteotoxicity and calreticulin surface exposure, instigating a series of immune responses including DC maturation, CD^{8+} T cell proliferation, and cytotoxic cytokine secretion (112, 125, 155, 200).

In head and neck tumors, several reports are using PDT as a treatment option, from early-stage tumors to those without other therapeutic alternatives. Complete remission rates range from 68% to 95% (201). According to the open-label phase 2 trial (NCT00453336), the PDT protocol employing the Photofrin™ evolved a better clinical outcome regarding the lesion location, the histopathological and clinical staging, thereby, less aggressive/invasive lesions located in the oral cavity at early stages of the disease. Briefly, the casuistic comprised 45 patients showing lesions in the oral cavity (53.3%), larynx (40%), or other lesions (6.7%). Concerning the histological subtypes, 22/45 related to squamous carcinoma, 13/45 to severe dysplasia, 9/45 *in situ* carcinomas, and 1/45 verrucous cancer. Upon a six month-based follow up, Photofrin™-PDT evolved completed cancer remission of 73% for less aggressive or invasive disease (e.g. severe dysplasia and *in situ* carcinoma), whereas it was of 50% for squamous carcinoma. On the other hand, clinical-stage squamous carcinoma (stage I) revealed 70% of complete responses, while more advanced stages obtained lower complete responses (i.e., 38%). This study counted to 45 adverse events, such as pain inside the oral cavity (53%) or moderate skin irritation (18%). Remarkably, after seven years of follow-up, 71% of patients obtained a desirable outcome; meanwhile, fewer patients required endoscopic resection (13%). Despite the difficulty of adequately managing the light device to provide proper dosimetry assessment, by following standard guidelines, it is possible to successfully tackle carcinomas at early stages, which after Photofrin™-PDT treatment, show cure rates in the oral cavity and larynx as high as 94% and 91%, respectively (202). Based on preclinical findings the tumor-resistance should decrease following Photofrin™-PDT combined with negative regulators of autophagy (e.g. 3-MA or BAF-A1) (161, 162).

The nonrandomized prospective clinical trial (NCT00530088) proposed to determine the efficacy of Photofrin™-PDT in the treatment for dysplasia, *in situ* carcinoma, or stage I carcinoma in the oral cavity and larynx. After following the patients for a mean period of 15 months, a significant and complete lesion remission was observed in 92% of the patients, with recurrence in only 13% of the treated cases. Adverse effects associated with PDT were transient local edema, pain, and phototoxic reaction (203). These findings corroborate with the PDT´s premise as an alternative and efficient strategy to treat cancers of the oral cavity and larynx since the protocols are already better defined.

HAL-PDT was investigated to treat cervical intraepithelial neoplasia of 262 patients during a randomized phase 2 clinical trial (NCT01256424) (204). In this study, 118 were diagnosed with CIN1 (low-grade squamous intraepithelial lesion), 83 with NIC2 (high-grade squamous intraepithelial lesion), the others were not considered as eligible for the study (i.e., NIC 3 or regular exam). Among those eligible, the frequency for high-risk oncogenic HPV (e.g. HPV 16/18) was 46% (6/13) and 37% (7/19) for CIN2 and CIN1, respectively. Aside from the significant and sustained tumor remission of 95% for CIN2, PDT also leads to a remarkable reduction of HPV infection. For instance, PDT undergoes clearance of high-risk HPV in 83% of CIN2 patients (5/6) compared to the control group (33%). Despite the favorable PDT response, adverse effects were reported in 125 patients and included vaginal discharge, local discomfort, and mild bleeding (204). In a phase 2 study (NCT00708942) involving 83 women diagnosed with CIN1, it was observed that the HAL-PDT was able to offer a complete cytohistological and HPV viral clearance in 90% of the patients, in a 6-month follow-up.

Even with PDT's mechanism inactivates the HPV virus is elusive; it seems to be related to the host's immune response. Studies suggest that there is an influence of antitumor immunity after PDT. This premise is based on experimental findings demonstrating the activation of dendritic cells and tumor-specific T response upon PDT. Beyond this immunity activation, PDT also triggers systemic inflammation causing oxidative damage and cytokines release (187, 188).

Studies carried out on lung tumors have shown that PDT might reduce airway obstruction and improve respiratory function (205, 206). The literature has reported several clinical studies highlighting PDT as a promising strategy to treat early-stage, superficial lung cancer, through a robotic transthoracic needle, and navigation bronchoscopy (205). The first clinical trial was conducted in 1993, through a prospective phase 2 trial on PDT using Photofrin II in which 84.8% cases of squamous cell carcinoma, centrally located, evolved a complete response after initial PDT-treatment (207). This favorable outcome extended for a median of 14 months (range 2-32 months). Aside from a lower frequency of side effects (e.g. photosensitivity in 2% of cases), PDT led to fewer cases of local recurrence in 4/50 (8%) cases during the 16-month follow-up. The multicenter phase 2 trial applying NPe6-PDT revealed a considerable outcome in patients succumbing with early-stage, lung squamous carcinoma (208). Again, PDT leads to a complete response in 85% of lesions but now with incredibly low skin photosensitivity. Based on these favorable findings, PDT with Photofrin II or NPe6 was approved in Japan as a suitable treatment for early-stage lung cancer, centrally located (205).

The uncontrolled, non-randomized, open-label, prospective, multicenter, phase 1 clinical trial (NCT03344861), performed in 10 patients, evaluated the safety of the tissue response to hematoporphyrin-PDT in solid lung tumor, previous to surgery. On the 15th day after PDT, patients were submitted to standard surgery, following macro and microscopic cancer evaluations. Despite the occurrence of side effects in 40% of patients, including hemorrhagic shock, anemia, and skin photosensitivity, the performance status and presence of inflammation suggest Photofrin™-PDT as a preoperative possibility in solid lung tumors. The same group conducted a phase 1, interventionist study (NCT02916745), in 5 patients diagnosed with non-small cell lung cancer or with lung metastasis. The objective was to assess the safety and viability of Photofrin™-iPDT by bronchoscopy intervention. The tumor remission with antitumor immunity after 6 months of iPDT was

complete (20%) or partial (60%). In general, this PDT-mediated antitumor-immunity has been associated with the activation of dendritic and T cells (188).

The critical role of autophagy in cell biology and its considerable therapeutic potential against cancer recently received the oncology community's attention. Autophagy generally promotes resistance to photodynamic therapy-induced apoptosis or necrosis and may serve as a strategy to improve its efficacy (129, 161). In line with this notion, PDT's autophagy modulation would represent a potential therapeutic target for human cancer (209, 210). We must be aware that the PDT-phototoxicity as well as the type of autophagy induction is dose-dependent, if cytoprotective or pro-death, as discussed earlier.

MAJOR CHALLENGES AND PERSPECTIVES

The trajectory of clinical PDT for cancer treatment is somewhat peculiar and not straightforward. Many new photosensitizers have been designed and tested, showing relatively important improvements compared to preceding ones. However, few of them were approved by the FDA and others are undergoing clinical trials (17). Despite the FDA approval, until now no PS presented a magic bullet or exhibited all characteristics of an ideal PS. Photofrin™, Foscan®, ALA and HAL are still the most used photosensitizers in PDT, despite the several disadvantages presented by them (17).

Thus, somehow a significant part of the knowledge acquired is not reaching clinical protocols routinely. It is essential to call attention to this fact and ask: how can we get through it? We consider that one of the bottlenecks for expanding the PDT application in a clinical routine because of the biological system's complexity. Toward this end, it would be necessary to stress the interaction of the PDT response regarding other intrinsic stressors, including cell stemness capacity, metabolic condition, cross-talking with the microenvironment and stroma, microbiota, genome instability, inflammatory and immune responses, vasculogenic mimicry, hypoxia, and other biochemical anomalies.

Another pivotal point comprises the discrepancy or even lack of a consensual or gold-standard procedure for PDT clinical practice concerning light dose exposure regimen, PS concentration, and the type of light device used. Also, topical products containing PS are lacking information about the exact amount applied to the skin and the time to be activated. All these points make it difficult to establish standard protocols that can be replicated in other studies. Furthermore, the progression-free survival investigation of clinical cases is still missing. We should also look for the PDT's ability to prolong the patient's time of life, instead of PDT being indicated only for the curative aspect.

Considering the premise that autophagy represents a therapeutic target to improve oncology clinical outcomes, future efforts should be made to the development of drugs with increased pharmacologic specificity beyond those commonly used in the current approach. Among all efforts, we have elicited mainly those focused on the development of novel autophagy inhibitors, whose consolidation into therapeutic regimens should be considered as a new avenue for the PDT antitumor field.

CONCLUDING REMARKS

In this review, we discussed the significant progress in the comprehension of autophagy modulation in cells succumbing to photooxidative damage. Preclinical reports pointed out that autophagy targeting can be a key regulatory routine to improve clinical outcomes in oncology practice. The repurposing drugs have been considered, including mTORC1 inhibitors (e.g. temsirolimus, everolimus, and rapamycin), chloroquine, and BAF-A1. Several efforts have been made to deal with tumor resistance. Chemical or photochemical inhibition of lysosomal function seems to bear a promising strategy since autophagy machinery plays a pivotal role in tumor vulnerability. Towards the increase in the death-autophagy related to lysosomal photodamage (e.g. verteporfin-PDT) or ER-stress (e.g. MPPa-PDT), the positive regulation of autophagy (e.g. BEZ235, LY-294002, or rapamycin) is highlighted as a promisor way to deal with tumor resistance. Notably, the modulation of parallel photodamage in lysosomes and mitochondria is a favorable route to trigger AACD thoroughly. Therefore, the PDT-mediated autophagy associated-cell death may be considered as a new therapeutic avenue, even though it needs to be further explored in clinical trials.

AUTHOR CONTRIBUTIONS

WM revised literature, organized and took the lead in writing the main manuscript with contribution from all authors. RB revised the literature and wrote mainly the section about the clinical outcome in cancer mediated by PDT. MNS contributed to the section about regulated cell death mechanisms PDT-induced in cancer cells and clinical outcome in cancer mediated by PDT. DG contributed to writing the section about tumor resistance. MS and TL contributed to describing the procedure and photophysical mechanism in PDT. RI and MB contributed to the overall review mainly about PDT concepts. TT also revised literature, wrote the PDT section, and contributed to the overall manuscript. WM and TT coordinated all the work. All authors contributed to the article and approved the submitted version.

FUNDING

The authors thank to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for grant numbers 2013/07937-8, 2016/23071-9, 2016/07642-6, and 2018/22922-0. Authors also acknowledge Programa de Apoio à Pós-graduação (PROAP) from Federal University of Uberlândia for financial support and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) – Finance Code 001.

ACKNOWLEDGMENTS

The authors are grateful to Research and Postgraduation Dean's Office from Federal University of Uberlândia (UFU) and Postgraduate Program of Chemistry Institute (IQUFU) for overall support and financial assistance. MB and RI are recipients from Conselho Nacional de Pesquisa (CNPq) research fellowships.

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin* (2020) 70:7–30. doi: 10.3322/caac.21590
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* (2018) 68:394–424. doi: 10.3322/caac.21492
3. Global Health Estimates Technical Paper WHO/HIS/IER/GHE/2018.3. *Global Health Estimates 2016: Disease burden by Cause, Age, Sex, by Country and by Region, 2000-2016*. Geneva: World Health Organization (WHO (2018).
4. Suzman RJB. *Global Health and Aging*. National Institute on Aging, National Institutes of Health, U.S. Department of Health and Human Services. World Health Organization WHO (2018).
5. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. *PloS Med* (2006) 3:2011–30. doi: 10.1371/journal.pmed.0030442
6. Dolmans DEJGJ, Fukumura D, Jain RK. TIMELINE: Photodynamic therapy for cancer. *Nat Rev Cancer* (2003) 3:380–7. doi: 10.1038/nrc1071
7. Perussi JR. Inativação fotodinâmica de microrganismos. *Quim Nova* (2007) 30:988–94. doi: 10.1590/S0100-40422007000400039
8. Varol M. An Alternative Treatment Modality of Diseases using Photodynamic Therapy with a Wide Range Biological Targeting Possibility. *Res Rev J Biol* (2015) 3:21–5.
9. Foote CS. Type I and type II mechanisms of photodynamic action. In: *Light-Activated Pesticides. ACS Symposium Series*. p. 22–38. doi: 10.1021/bk-1987-0339.ch002
10. Ormond AB, Freeman HS. Dye sensitizers for photodynamic therapy. *Mater (Basel)* (2013) 6:817–40. doi: 10.3390/ma6030817
11. Baptista MS, Cadet J, Di Mascio P, Ghogare AA, Greer A, Hamblin MR, et al. Type I and II Photosensitized Oxidation Reactions: Guidelines and Mechanistic Pathways. *Photochem Photobiol* (2017) 38:42–9. doi: 10.1111/php.12716
12. Tsubone TM, Baptista MS, Itri R. Understanding membrane remodelling initiated by photosensitized lipid oxidation. *Biophys Chem* (2019) 254:106263. doi: 10.1016/j.bpc.2019.106263
13. Foote CS. Definition of Type I and Type II Photosensitized Oxidation. *Photochem Photobiol* (1991) 54:659. doi: 10.1111/j.1751-1097.1991.tb02071.x
14. Mokwena MG, Kruger CA, Ivan MT, Heidi A. A review of nanoparticle photosensitizer drug delivery uptake systems for photodynamic treatment of lung cancer. *Photodiagn Photodyn Ther* (2018) 22:147–54. doi: 10.1016/j.pdpdt.2018.03.006
15. Kim MM, Darafsheh A. Light Sources and Dosimetry Techniques for Photodynamic Therapy. *Photochem Photobiol* (2020) 96:280–94. doi: 10.1111/php.13219
16. Davanzo NN, Pellosi DS, Franchi LP, Tedesco AC. Light source is critical to induce glioblastoma cell death by photodynamic therapy using chloro-aluminiumphtalocyanine albumin-based nanoparticles. *Photodiagn Photodyn Ther* (2017) 19:181–3. doi: 10.1016/j.pdpdt.2017.04.017
17. Hamblin MR. Photodynamic Therapy for Cancer: What's Past is Prologue. *Photochem Photobiol* (2020) 96:506–16. doi: 10.1111/php.13190
18. Isele U, Van Hoogevest P, Capraro H -G, Schieweck K, Hilfiker R, Leuenberger H. Large-scale production of liposomes containing monomeric zinc phthalocyanine by controlled dilution of organic solvents. *J Pharm Sci* (1994) 83:1608–16. doi: 10.1002/jps.2600831117
19. Mroz P, Yaroslavsky A, Kharkwal GB, Hamblin MR. Cell death pathways in photodynamic therapy of cancer. *Cancers (Basel)* (2011) 3:2516–39. doi: 10.3390/cancers3022516
20. Rodgers MAJ, Snowden PT. Lifetime of oxygen (O2(1.DELTA.g)) in liquid water as determined by time-resolved infrared luminescence measurements. *J Am Chem Soc* (1982) 104:5541–3. doi: 10.1021/ja00384a070
21. Redmond RW, Kochevar IE. Spatially Resolved Cellular Responses to Singlet Oxygen. *Photochem Photobiol* (2006) 82:1178–86. doi: 10.1562/2006-04-14-1R-874
22. Oliveira CS, Turchiello R, Kowaltowski AJ, Indig GL, Baptista MS. Major determinants of photoinduced cell death: Subcellular localization versus photosensitization efficiency. *Free Radic Biol Med* (2011) 51:824–33. doi: 10.1016/j.freeradbiomed.2011.05.023
23. Girotti AW. Photosensitized oxidation of membrane lipids: reaction pathways, cytotoxic effects, and cytoprotective mechanisms. *J Photochem Photobiol B* (2001) 63:103–13. doi: S101113440100207X [pii doi: 10.1016/S1011-1344(01)00207-X
24. Henderson BW, Dougherty TJ. How Does Photodynamic Therapy Work. *Photochem Photobiol* (1992) 55:145–57. doi: 10.1111/j.1751-1097.1992.tb04222.x
25. Joshi P, Ethirajan M, Goswami LN, Srivatsan A, Missert JR, Pandey RK. Synthesis, spectroscopic, and in vitro photosensitizing efficacy of ketobacteriochlorins derived from ring-B and ring-D reduced chlorins via pinacol-pinacolone rearrangement. *J Org Chem* (2011) 76:8629–40. doi: 10.1021/jo201688c
26. Pereira MM, Monteiro CJP, Simões AVC, Pinto SMA, Abreu AR, Sá GFF, et al. Synthesis and photophysical characterization of a library of photostable halogenated bacteriochlorins: An access to near infrared chemistry. *Tetrahedron* (2010) 66:9545–51. doi: 10.1016/j.tet.2010.09.106
27. Zheng G, Potter WR, Camacho SH, Missert JR, Wang G, Bellnier DA, et al. Synthesis, photophysical properties, tumor uptake, and preliminary in vivo photosensitizing efficacy of a homologous series of 3-(1′-alkyloxy)-ethyl-3-devinylpurpurin-18-N-alkylimides with variable lipophilicity. *J Med Chem* (2001) 44:1540–59. doi: 10.1021/jm0005510
28. Pereira MM, Monteiro CJP, Simões AVC, Pinto SMA, Arnaut LG, Sá GFF, et al. Synthesis and photophysical properties of amphiphilic halogenated bacteriochlorins: new opportunities for photodynamic therapy of cancer. *J Porphyr Phthalocyanines* (2009) 13:567–73. doi: 10.1142/S1088424609000553
29. Pavani C, Uchoa AF, Oliveira CS, Iamamoto Y, Baptista MS. Effect of zinc insertion and hydrophobicity on the membrane interactions and PDT activity of porphyrin photosensitizers. *Photochem Photobiol Sci* (2009) 8:233–40. doi: 10.1039/b810313e
30. Uchoa AF, Oliveira CS, Baptista MS. Relationship between structure and photoactivity of porphyrins derived from protoporphyrin IX. *J Porphyr Phthalocyanines* (2010) 14:832–45. doi: 10.1142/S108842461000263X
31. Uchoa AF, De Oliveira KT, Baptista MS, Bortoluzzi AJ, Iamamoto Y, Serra OA. Chlorin photosensitizers sterically designed to prevent self-aggregation. *J Org Chem* (2011) 76:8824–32. doi: 10.1021/jo201568n
32. Guidotti G. Membrane proteins. *Annu Rev Biochem* (1972) 41:731–52. doi: 10.1146/annurev.bi.41.070172.003503
33. Brien JSO. Cell Membranes- Composition: Structure: Function. *J Theor Biol* (1967) 15:307–24. doi: 10.1016/0022-5193(67)90140-3
34. Engelmann FM, Mayer I, Gabrielli DS, Toma HE, Kowaltowski AJ, Araki K, et al. Interaction of cationic meso-porphyrins with liposomes, mitochondria and erythrocytes. *J Bioenerg Biomembr* (2007) 39:175–85. doi: 10.1007/s10863-007-9075-0
35. Tsubone TM, Martins WK, Pavani C, Junqueira HC, Itri R, Baptista MS. Enhanced efficiency of cell death by lysosome-specific photodamage. *Sci Rep* (2017) 7:6734. doi: 10.1038/s41598-017-06788-7
36. Bacellar IOLL, Pavani C, Sales EM, Itri R, Wainwright M, Baptista MS. Membrane damage efficiency of phenothiazinium photosensitizers. *Photochem Photobiol* (2014) 90:801–13. doi: 10.1111/php.12264

37. Pavani C, Iamamoto Y, Baptista MS. Mechanism and efficiency of cell death of type II photosensitizers: Effect of zinc chelation. *Photochem Photobiol* (2012) 88:774–81. doi: 10.1111/j.1751-1097.2012.01102.x
38. Ricchelli F, Franchi L, Miotto G, Borsetto L, Gobbo S, Nikolov P, et al. Meso-substituted tetra-cationic porphyrins photosensitize the death of human fibrosarcoma cells via lysosomal targeting. *Int J Biochem Cell Biol* (2005) 37:306–19. doi: 10.1016/j.biocel.2004.06.013
39. Tasso TT, Tsubone TM, Baptista MS, Mattiazzi LM, Acunha TV, Iglesias BA. Isomeric effect on the properties of tetraplatinated porphyrins showing optimized phototoxicity for photodynamic therapy. *Dalton Trans* (2017) 46 (33):11037–45. doi: 10.1039/C7DT01205E
40. Davies MJ. Singlet oxygen-mediated damage to proteins and its consequences. *Biochem Biophys Res Commun* (2003) 305:761–70. doi: 10.1016/S0006-291X(03)00817-9
41. Baker A, Kanofsky JR. Quenching of singlet oxygen by biomolecules from L1210 leukemia cells. *Photochem Photobiol* (1992) 55:523–8. doi: 10.1111/j.1751-1097.1992.tb04273.x
42. Baptista MS, Indig GL. Effect of BSA binding on photophysical and photochemical properties of triarylmethane dyes. *J Phys Chem B* (1998) 102:4678–88. doi: 10.1021/jp981185n
43. Sen S, Sett R, Paul BK, Guchhait N. Interaction of phenazinium-based photosensitizers with the 'N' and 'B' isoforms of human serum albumin: Effect of methyl substitution. *J Photochem Photobiol B Biol* (2017) 174:217–28. doi: 10.1016/j.jphotobiol.2017.08.002
44. Cozzolino M, Delcanale P, Montali C, Tognolini M, Giorgio C, Corrado M, et al. Enhanced photosensitizing properties of protein bound curcumin. *Life Sci* (2019) 233:116710. doi: 10.1016/j.lfs.2019.116710
45. Chakrabortty S, Agrawalla BK, Stumper A, Vegi NM, Fischer S, Reichardt C, et al. Mitochondria Targeted Protein-Ruthenium Photosensitizer for Efficient Photodynamic Applications. *J Am Chem Soc* (2017) 139:2512–9. doi: 10.1021/jacs.6b13399
46. St Denis TG, Hamblin MR. Synthesis, bioanalysis and biodistribution of photosensitizer conjugates for photodynamic therapy. *Bioanalysis* (2013) 5:1099–114. doi: 10.4155/bio.13.37
47. Bacellar I, Tsubone T, Pavani C, Baptista M. Photodynamic Efficiency: From Molecular Photochemistry to Cell Death. *Int J Mol Sci* (2015) 16:20523–59. doi: 10.3390/ijms160920523
48. Savellano MD, Hasan T. Targeting Cells That Overexpress the Epidermal Growth Factor Receptor with Polyethylene Glycolated BPD Verteporfin Photosensitizer Immunoconjugates. *Photochem Photobiol* (2003) 77:431. doi: 10.1562/0031-8655(2003)077<0431:tctote>2.0.co;2
49. Abu-Yousif AO, Moor ACE, Zheng X, Savellano MD, Yu W, Selbo PK, et al. Epidermal Growth Factor Receptor-Targeted Photosensitizer Selectively Inhibits EGFR Signaling and Induces Targeted Phototoxicity In Ovarian Cancer Cells. *Cancer Lett* (2012) 321:120–7. doi: 10.1038/jid.2014.371
50. Mitsunaga M, Ogawa M, Kosaka N, Rosenblum LT, Choyke PL, Kobayashi H. Cancer cell-selective in vivo near infrared photoimmunotherapy targeting specific membrane molecules. *Nat Med* (2011) 17:1685–91. doi: 10.1038/nm.2554
51. Kercher EM, Nath S, Rizvi I, Spring BQ. Cancer Cell-targeted and Activatable Photoimmunotherapy Spares T Cells in a 3D Coculture Model. *Photochem Photobiol* (2020) 96:295–300. doi: 10.1111/php.13153
52. Sato K, Sato N, Xu B, Nakamura Y, Nagaya T, Choyke PL, et al. Spatially selective depletion of tumor-associated regulatory T cells with near-infrared photoimmunotherapy. *Sci Transl Med* (2016) 8(352):352ra110. doi: 10.1126/scitranslmed.aaf6843
53. Zhen Z, Tang W, Wang M, Zhou S, Wang H, Wu Z, et al. Protein Nanocage Mediated Fibroblast-Activation Protein Targeted Photoimmunotherapy to Enhance Cytotoxic T Cell Infiltration and Tumor Control. *Nano Lett* (2017) 17:862–9. doi: 10.1021/acs.nanolett.6b04150
54. Lihuan D, Jingcun Z, Ning J, Guozeng W, Yiwei C, Wei L, et al. Photodynamic therapy with the novel photosensitizer chlorophyllin f induces apoptosis and autophagy in human bladder cancer cells. *Lasers Surg Med* (2014) 46:319–34. doi: 10.1002/lsm.22225
55. Du L, Jiang N, Wang G, Chu Y, Lin W, Qian J, et al. Autophagy inhibition sensitizes bladder cancer cells to the photodynamic effects of the novel photosensitizer chlorophyllin e4. *J Photochem Photobiol B* (2014) 133:1–10. doi: 10.1016/j.jphotobiol.2014.02.010
56. François A, Marchal S, Guillemin F, Bezdetnaya L. mTHPC-based photodynamic therapy induction of autophagy and apoptosis in cultured cells in relation to mitochondria and endoplasmic reticulum stress. *Int J Oncol* (2011) 39:1537–43. doi: 10.3892/ijo.2011.1174
57. Inguscio V, Panzarini E, Dini L. Autophagy Contributes to the Death/Survival Balance in Cancer PhotoDynamic Therapy. *Cells* (2012) 1:464–91. doi: 10.3390/cells1030464
58. Rubio NN, Coupienne I, Di Valentin E, Heirman I, Grooten J, Piette J, et al. Spatiotemporal autophagic degradation of oxidatively damaged organelles after photodynamic stress is amplified by mitochondrial reactive oxygen species. *Autophagy* (2012) 8:1312–24. doi: 10.4161/auto.20763
59. Martins WK, Santos NF, Rocha C de S, Bacellar IOL, Tsubone TM, Viotto AC, et al. Parallel damage in mitochondria and lysosomes is an efficient way to photoinduce cell death. *Autophagy* (2019) 15:259–79. doi: 10.1080/15548627.2018.1515609
60. Dewaele M, Martinet W, Rubio N, Verfaillie T, de Witte PA, Piette J, et al. Autophagy pathways activated in response to PDT contribute to cell resistance against ROS damage. *J Cell Mol Med* (2011) 15:1402–14. doi: 10.1111/j.1582-4934.2010.01118.x
61. Reiners JJ, Agostinis P, Berg K, Oleinick NL, Kessel D. Assessing autophagy in the context of photodynamic therapy. *Autophagy* (2010) 6:7–18. doi: 10.4161/auto.6.1.10220
62. Zhu J, Tian S, Li KT, Chen Q, Jiang Y, Lin HD, et al. Inhibition of breast cancer cell growth by methyl pyropheophenylchlorin photodynamic therapy is mediated though endoplasmic reticulum stress-induced autophagy in vitro and vivo. *Cancer Med* (2018) 7:1908–20. doi: 10.1002/cam4.1418
63. Kessel D. Autophagic death probed by photodynamic therapy. *Autophagy* (2015) 11:1941–3. doi: 10.1080/15548627.2015.1078960
64. Anding AL, Baehrecke EH. Cleaning House: Selective Autophagy of Organelles. *Dev Cell* (2017) 41:10–22. doi: 10.1016/j.devcel.2017.02.016
65. de Faria CMG, Inada NM, Vollet-Filho JD, Bagnato VS. A threshold dose distribution approach for the study of PDT resistance development: A threshold distribution approach for the study of PDT resistance. *J Photochem Photobiol B Biol* (2018) 182:85–91. doi: 10.1016/j.jphotobiol.2018.03.022
66. Kessel D, Oleinick NL. Cell Death Pathways Associated with Photodynamic Therapy: An Update. *Photochem Photobiol* (2018) 94:213–8. doi: 10.1111/php.12857
67. Biteghe FN, Davids LM. A combination of photodynamic therapy and chemotherapy displays a differential cytotoxic effect on human metastatic melanoma cells. *J Photochem Photobiol B Biol* (2017) 166:18–27. doi: 10.1016/j.jphotobiol.2016.11.004
68. Zhang L, Ji Z, Zhang J, Yang S. Photodynamic therapy enhances skin cancer chemotherapy effects through autophagy regulation. *Photodiagn Photodyn Ther* (2019) 28:159–65. doi: 10.1016/j.pdpdt.2019.08.023
69. Li H, Liu C, Zeng YP, Hao YH, Huang JW, Yang ZY, et al. Nanoceria-mediated drug delivery for targeted photodynamic therapy on drug-resistant breast cancer. *ACS Appl Mater Interfaces* (2016) 8:31510–23. doi: 10.1021/acsami.6b07338
70. Shi L, Hu F, Duan Y, Wu W, Dong J, Meng X, et al. Hybrid Nanospheres to Overcome Hypoxia and Intrinsic Oxidative Resistance for Enhanced Photodynamic Therapy. *ACS Nano* (2020) 14:2183–90. doi: 10.1021/acsnano.9b09032
71. Liu X, Fan Z, Zhang L, Jin Z, Yan D, Zhang Y, et al. Bcl-2 inhibitor uploaded upconversion nanophotosensitizers to overcome the photodynamic therapy resistance of cancer through adjuvant intervention strategy. *Biomaterials* (2017) 144:73–83. doi: 10.1016/j.biomaterials.2017.08.010
72. Li W, Guo X, Kong F, Zhang H, Luo L, Li Q, et al. Overcoming photodynamic resistance and tumor targeting dual-therapy mediated by indocyanine green conjugated gold nanospheres. *J Control Release* (2017) 258:171–81. doi: 10.1016/j.jconrel.2017.05.015
73. Zamarrón A, Lucena SR, Salazar N, Sanz-Rodríguez F, Jaén P, Gilaberte Y, et al. Isolation and characterization of PDT-resistant cancer cells. *Photochem Photobiol Sci* (2015) 14:1378–89. doi: 10.1039/c4pp00448e
74. Valli F, García Vior MC, Roguin LP, Marino J. Crosstalk between oxidative stress-induced apoptotic and autophagic signaling pathways in Zn(II) phthalocyanine photodynamic therapy of melanoma. *Free Radic Biol Med* (2020) 152:743–54. doi: 10.1016/j.freeradbiomed.2020.01.018

75. Casas A, Di Venosa G, Hasan T, Batlle A. Mechanisms of Resistance to Photodynamic Therapy. *Curr Med Chem* (2011) 18:2486–515. doi: 10.2174/092986711795843272
76. Olsen CE, Weyergang A, Edwards VT, Berg K, Brech A, Weisheit S, et al. Development of resistance to photodynamic therapy (PDT) in human breast cancer cells is photosensitizer-dependent: Possible mechanisms and approaches for overcoming PDT-resistance. *Biochem Pharmacol* (2017) 144:63–77. doi: 10.1016/j.bcp.2017.08.002
77. Liu J, Kang R, Tang D. ESCRT-III-mediated membrane repair in cell death and tumor resistance. *Cancer Gene Ther* (2020) 1–4. doi: 10.1038/s41417-020-0200-0
78. Yea SS, Fruman DA. Achieving cancer cell death with PI3K/mTOR-targeted therapies. *Ann N Y Acad Sci* (2013) 1280(1):15–8. doi: 10.1111/nyas.12028
79. Chizenga EP, Abrahamse H. Nanotechnology in modern photodynamic therapy of cancer: A review of cellular resistance patterns affecting the therapeutic response. *Pharmaceutics* (2020) 12:1–21. doi: 10.3390/pharmaceutics12070632
80. Steinbichler TB, Dudás J, Skvortsov S, Ganswindt U, Riechelmann H, Skvortsova II. Therapy resistance mediated by cancer stem cells. *Semin Cancer Biol* (2018) 53:156–67. doi: 10.1016/j.semcancer.2018.11.006
81. Di Venosa G, Perotti C, Batlle A, Casas A. The role of cytoskeleton and adhesion proteins in the resistance to photodynamic therapy. Possible therapeutic interventions. *Photochem Photobiol Sci* (2015) 14:1451–64. doi: 10.1039/c4pp00445k
82. Lamberti MJ, Morales Vasconsuelo AB, Ferrara MG, Rumie Vittar NB. Recapitulation of Hypoxic Tumor–stroma Microenvironment to Study Photodynamic Therapy Implications. *Photochem Photobiol* (2020) 96:897–905. doi: 10.1111/php.13220
83. Lamberti MJ, Pansa MF, Vera RE, Fernández-Zapico ME, Vittar NBR, Rivarola VA. Transcriptional activation of HIF-1 by a ROSERK axis underlies the resistance to photodynamic therapy. *PloS One* (2017) 12:1–16. doi: 10.1371/journal.pone.0177801
84. Rodríguez ME, Catrinacio C, Ropolo A, Rivarola VA, Vaccaro MI. A novel HIF-1α/VMP1-autophagic pathway induces resistance to photodynamic therapy in colon cancer cells. *Photochem Photobiol Sci* (2017) 16:1631–42. doi: 10.1039/c7pp00161d
85. Stacy AE, Jansson PJ, Richardson DR. Molecular Pharmacology of ABCG2 and its role in chemoresistance. *Mol Pharmacol* (2013) 84:655–69. doi: 10.1124/mol.113.088609
86. Kralova J, Kolar M, Kahle M, Truksa J, Lettlova S, Balusikova K, et al. Glycol porphyrin derivatives and temoporfin elicit resistance to photodynamic therapy by different mechanisms. *Sci Rep* (2017) 7:1–15. doi: 10.1038/srep44497
87. Jendželovský R, Jendželovská Z, Kuchárová B, Fedoročko P. Breast cancer resistance protein is the enemy of hypericin accumulation and toxicity of hypericin-mediated photodynamic therapy. *BioMed Pharmacother* (2019) 109:2173–81. doi: 10.1016/j.biopha.2018.11.084
88. Baglo Y, Liang BJ, Robey RW, Ambudkar SV, Gottesman MM, Huang HC. Porphyrin-lipid assemblies and nanovesicles overcome ABC transporter-mediated photodynamic therapy resistance in cancer cells. *Cancer Lett* (2019) 457:110–8. doi: 10.1016/j.canlet.2019.04.037
89. Robey RW, Steadman K, Polgar O, Bates SE. ABCG2-mediated transport of photosensitizers. *Cancer Biol Ther* (2005) 4:187–94. doi: 10.4161/cbt.4.2.1440
90. Hira D, Terada T. BCRP/ABCG2 and high-alert medications: Biochemical, pharmacokinetic, pharmacogenetic, and clinical implications. *Biochem Pharmacol* (2018) 147:201–10. doi: 10.1016/j.bcp.2017.10.004
91. Hagiya Y, Fukuhara H, Matsumoto K, Endo Y, Nakajima M, Tanaka T, et al. Expression levels of PEPT1 and ABCG2 play key roles in 5-aminolevulinic acid (ALA)-induced tumor-specific protoporphyrin IX (PpIX) accumulation in bladder cancer. *Photodiagn Photodyn Ther* (2013) 10:288–95. doi: 10.1016/j.pdpdt.2013.02.001
92. Chekwube AE, George B, Abrahamse H. Phototoxic effectiveness of zinc phthalocyanine tetrasulfonic acid on MCF-7 cells with overexpressed P-glycoprotein. *J Photochem Photobiol B Biol* (2020) 204:111811. doi: 10.1016/j.jphotobiol.2020.111811
93. Mastrangelopoulou M, Grigalavicius M, Raabe TH, Skarpen E, Juzenas P, Peng Q, et al. Predictive biomarkers for 5-ALA-PDT can lead to personalized treatments and overcome tumor-specific resistances. *Cancer Rep* (2020) 1–16:e1278. doi: 10.1002/cnr2.1278
94. Zhang X, Chen X, Guo Y, Jia HR, Jiang YW, Wu FG. Endosome/lysosome-detained supramolecular nanogels as an efflux retarder and autophagy inhibitor for repeated photodynamic therapy of multidrug-resistant cancer. *Nanoscale Horizons* (2020) 5:481–7. doi: 10.1039/c9nh00643e
95. La Belle Flynn A, Calhoun BC, Sharma A, Chang JC, Almasan A, Schiemann WP. Autophagy inhibition elicits emergence from metastatic dormancy by inducing and stabilizing Pfkfb3 expression. *Nat Commun* (2019) 10:1–15. doi: 10.1038/s41467-019-11640-9
96. Gotink KJ, Broxterman HJ, Labots M, de Haas RR, Dekker H, Honeywell RJ, et al. Lysosomal Sequestration of Sunitinib: A Novel Mechanism of Drug Resistance. *Clin Cancer Res* (2011) 17:7337–46. doi: 10.1158/1078-0432.CCR-11-1667
97. Tan Q, Joshua AM, Wang M, Bristow RG, Wouters BG, Allen CJ, et al. Up-regulation of autophagy is a mechanism of resistance to chemotherapy and can be inhibited by pantoprazole to increase drug sensitivity. *Cancer Chemother Pharmacol* (2017) 79:959–69. doi: 10.1007/s00280-017-3298-5
98. Zhao SG, Chen XF, Wang LG, Yang G, Han DY, Teng L, et al. Increased expression of ABCB6 enhances protoporphyrin ix accumulation and photodynamic effect in human glioma. *Ann Surg Oncol* (2013) 20:4379–88. doi: 10.1245/s10434-011-2201-6
99. Nakayama T, Otsuka S, Kobayashi T, Okajima H, Matsumoto K, Hagiya Y, et al. Dormant cancer cells accumulate high protoporphyrin IX levels and are sensitive to 5-aminolevulinic acid-based photodynamic therapy. *Sci Rep* (2016) 6:1–6. doi: 10.1038/srep36478
100. Bhowmick R, Girotti AW. Cytoprotective signaling associated with nitric oxide upregulation in tumor cells subjected to photodynamic therapy-like oxidative stress. *Free Radic Biol Med* (2013) 57:39–48. doi: 10.1016/j.freeradbiomed.2012.12.005
101. Kim J, Lim W, Kim S, Jeon S, Hui Z, Ni K, et al. Photodynamic therapy (PDT) resistance by PARP1 regulation on PDT-induced apoptosis with autophagy in head and neck cancer cells. *J Oral Pathol Med* (2014) 43:675–84. doi: 10.1111/jop.12195
102. Xiong L, Liu Z, Ouyang G, Lin L, Huang H, Kang H, et al. Autophagy inhibition enhances photocytotoxicity of Photosan-II in human colorectal cancer cells. *Oncotarget* (2017) 8:6419–32. doi: 10.18632/oncotarget.14117
103. Coupienne I, Bontems S, Dewaele M, Rubio N, Habraken Y, Fulda S, et al. NF-kappaB inhibition improves the sensitivity of human glioblastoma cells to 5-aminolevulinic acid-based photodynamic therapy. *Biochem Pharmacol* (2011) 81:606–16. doi: 10.1016/j.bcp.2010.12.015
104. Ji H-T, Chien L-T, Lin Y-H, Chien H-F, Chen C-T. 5-ALA mediated photodynamic therapy induces autophagic cell death via AMP-activated protein kinase. *Mol Cancer* (2010) 9:91. doi: 10.1186/1476-4598-9-91
105. Oberdanner CB, Plaetzer K, Kiesslich T, Krammer B. Photodynamic Treatment with Fractionated Light Decreases Production of Reactive Oxygen Species and Cytotoxicity in vitro via Regeneration of Glutathione. *Photochem Photobiol* (2005) 81(3):609–13. doi: 10.1562/2004-08-23-RN-284
106. Wang HP, Hanlon JG, Rainbow AJ, Espiritu M, Singh G. Up-regulation of Hsp27 Plays a Role in the Resistance of Human Colon Carcinoma HT29 Cells to Photooxidative Stress. *Photochem Photobiol* (2002) 76:98. doi: 10.1562/0031-8655(2002)076<0098:urohpa>2.0.co;2
107. Rodríguez ME, Arévalo DE, Milla Sanabria L, Cuello Carrión FD, Fanelli MA, Rivarola VA. Heat shock protein 27 modulates autophagy and promotes cell survival after photodynamic therapy. *Photochem Photobiol Sci* (2019) 18:546–54. doi: 10.1039/c8pp00536b
108. Kim J, Lim H, Kim S, Cho H, Kim Y, Li X, et al. Effects of HSP27 downregulation on PDT resistance through PDT-induced autophagy in head and neck cancer cells. *Oncology Reports* (2016) 35(2016):2237–45. doi: 10.3892/or.2016.4597
109. Kim J, Jung H, Lim W, Kim S, Ko Y, Karna S, et al. Down-regulation of heat-shock protein 27-induced resistance to photodynamic therapy in oral cancer cells. *J Oral Pathol Med* (2013) 42:9–16. doi: 10.1111/j.1600-0714.2012.01155.x
110. Thomas DD. The biological lifetime of nitric oxide: Implications for the perivascular dynamics of NO and O2. *Proc Natl Acad Sci* (2001) 98:355–60. doi: 10.1073/pnas.011379598

111. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: Structure, function and inhibition. *Biochem J* (2001) 357:593–615. doi: 10.1042/0264-6021:3570593
112. Tsubone TM, Martins WK, Franco MSF, Silva MN, Itri R, Baptista MS. Cellular compartments challenged by membrane photo-oxidation. *Arch Biochem Biophys* (2021) 697:108665. doi: 10.1016/j.abb.2020.108665
113. Korbelik M, Parkins CS, Shibuya H, Cecic I, Stratford MRL, Chaplin DJ. Nitric oxide production by tumour tissue: Impact on the response to photodynamic therapy. *Br J Cancer* (2000) 82:1835–43. doi: 10.1054/bjoc.2000.1157
114. Bhowmick R, Girotti AW. Signaling events in apoptotic photokilling of 5-aminolevulinic acid-treated tumor cells: Inhibitory effects of nitric oxide. *Free Radic Biol Med* (2009) 47:731–40. doi: 10.1016/j.freeradbiomed.2009.06.009
115. Bhowmick R, Girotti AW. Cytoprotective induction of nitric oxide synthase in a cellular model of 5-aminolevulinic acid-based photodynamic therapy. *Free Radic Biol Med* (2010) 48:1296–301. doi: 10.1016/j.freeradbiomed.2010.01.040
116. Bhowmick R, Girotti AW. Rapid upregulation of cytoprotective nitric oxide in breast tumor cells subjected to a photodynamic therapy-like oxidative challenge. *Photochem Photobiol* (2011) 87:378–86. doi: 10.1111/j.1751-1097.2010.00877.x
117. Fahey JM, Girotti AW. Nitric oxide-mediated resistance to photodynamic therapy in a human breast tumor xenograft model: Improved outcome with NOS2 inhibitors. *Nitric Oxide - Biol Chem* (2017) 62:52–61. doi: 10.1016/j.niox.2016.12.003
118. Fahey JM, Emmer JV, Korytowski W, Hogg N, Girotti AW. Antagonistic Effects of Endogenous Nitric Oxide in a Glioblastoma Photodynamic Therapy Model. *Photochem Photobiol* (2016) 92:842–53. doi: 10.1111/php.12636
119. Fahey JM, Girotti AW. Nitric oxide antagonism to anti-glioblastoma photodynamic therapy: Mitigation by inhibitors of nitric oxide generation. *Cancers* (2019) 11(2):231–46. doi: 10.3390/cancers11020231
120. Bazak J, Fahey JM, Wawak K, Korytowski W, Girotti AW. Enhanced aggressiveness of bystander cells in an anti-tumor photodynamic therapy model: Role of nitric oxide produced by targeted cells. *Free Radic Biol Med* (2017) 102:111–21. doi: 10.1016/j.freeradbiomed.2016.11.034
121. Han X, Zhong Z, Kou J, Zheng Y, Liu Z, Jiang Y, et al. ROS generated by upconversion nanoparticle-mediated photodynamic therapy induces autophagy via PI3K/AKT/ mTOR signaling pathway in M1 peritoneal macrophage. *Cell Physiol Biochem* (2018) 48:1616–27. doi: 10.1159/000492283
122. Rosin FCP, Teixeira MG, Pelissari C, Corrêa L. Photodynamic Therapy Mediated by 5-aminolevulinic Acid Promotes the Upregulation and Modifies the Intracellular Expression of Surveillance Proteins in Oral Squamous Cell Carcinoma. *Photochem Photobiol* (2019) 95:635–43. doi: 10.1111/php.13029
123. Girotti AW. Upregulation of nitric oxide in tumor cells as a negative adaptation to photodynamic therapy. *Lasers Surg Med* (2018) 50:590–8. doi: 10.1002/lsm.22807
124. Molejon MI, Ropolo A, Lo R, Boggio V, Vaccaro MI. The VMP1-Beclin 1 interaction regulates autophagy induction. *Sci Rep* (2013) 3:1–11. doi: 10.1038/srep01055
125. Garg AD, Agostinis P. ER stress, autophagy and immunogenic cell death in photodynamic therapy-induced anti-cancer immune responses. *Photochem Photobiol Sci* (2014) 13:474–87. doi: 10.1039/c3pp50333j
126. Garg AD, Dudek AM, Ferreira GB, Verfaillie T, Vandenabeele P, Krysko DV, et al. ROS-induced autophagy in cancer cells assists in evasion from determinants of immunogenic cell death. *Autophagy* (2013) 9:1292–307. doi: 10.4161/auto.25399
127. Garg AD, Dudek AM, Agostinis P. Calreticulin surface exposure is abrogated in cells lacking, chaperone-mediated autophagy-essential gene, LAMP2A. *Cell Death Dis* (2013) 4:e826–2. doi: 10.1038/cddis.2013.372
128. Lin S, Yang L, Shi H, Du W, Qi Y, Qiu C, et al. Endoplasmic reticulum-targeting photosensitizer Hypericin confers chemo-sensitization towards oxaliplatin through inducing pro-death autophagy. *Int J Biochem Cell Biol* (2017) 87:54–68. doi: 10.1016/j.biocel.2017.04.001
129. Wei MF, Chen MW, Chen KC, Lou PJ, Lin SYF, Hung SC, et al. Autophagy promotes resistance to photodynamic therapy-induced apoptosis selectively in colorectal cancer stem-like cells. *Autophagy* (2014) 10:1179–92. doi: 10.4161/auto.28679
130. Orsi A, Razi M, Dooley HC, Robinson D, Weston AE, Collinson LM, et al. Dynamic and transient interactions of Atg9 with autophagosomes, but not membrane integration, are required for autophagy. *Mol Biol Cell* (2012) 23:1860–73. doi: 10.1091/mbc.e11-09-0746
131. Karanasios E, Walker SA, Okkenhaug H, Manifava M, Hummel E, Zimmermann H, et al. Autophagy initiation by ULK complex assembly vesicles. *Nat Commun* (2016) 7:1–17. doi: 10.1038/ncomms12420
132. Kim J, Kundu M, Viollet B, Guan K-L. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol* (2011) 13:132–41. doi: 10.1038/ncb2152
133. Egan DF, Shackelford DB, Mihaylova MM, Gelino S, Kohnz RA, Mair W, et al. Phosphorylation of ULK1 (hATG1) by AMP-Activated Protein Kinase Connects Energy Sensing to Mitophagy. *Sci (80-)* (2011) 331:456–61. doi: 10.1126/science.1196371
134. Itakura E, Mizushima N. Characterization of autophagosome formation site by a hierarchical analysis of mammalian Atg proteins. *Autophagy* (2010) 6:764–76. doi: 10.4161/auto.6.6.12709
135. Huang X, Chen J, Wu W, Yang W, Zhong B, Qing X, et al. Delivery of MutT homolog 1 inhibitor by functionalized graphene oxide nanoparticles for enhanced chemo-photodynamic therapy triggers cell death in osteosarcoma. *Acta Biomater* (2020) 109:229–43. doi: 10.1016/j.actbio.2020.04.009
136. Kong F, Zou H, Liu X, He J, Zheng Y, Xiong L, et al. miR-7112-3p targets PERK to regulate the endoplasmic reticulum stress pathway and apoptosis induced by photodynamic therapy in colorectal cancer CX-1 cells. *Photodiagn Photodyn Ther* (2020) 29:101663. doi: 10.1016/j.pdpdt.2020.101663
137. Kim I, Lemasters JJ. Mitophagy selectively degrades individual damaged mitochondria after photoirradiation. *Antioxid Redox Signal* (2011) 14:1919–28. doi: 10.1089/ars.2010.3768
138. Youle RJ, Narendra DP. Mechanisms of mitophagy. *Nat Rev Mol Cell Biol* (2011) 12:9–14. doi: 10.1038/nrm3028
139. Ding W-X, Yin X-M. Mitophagy: mechanisms, pathophysiological roles, and analysis. *Biol Chem* (2012) 393:547–64. doi: 10.1515/hsz-2012-0119
140. Lemasters JJ. Variants of mitochondrial autophagy: Types 1 and 2 mitophagy and micromitophagy (Type 3). *Redox Biol* (2014) 2:749–54. doi: 10.1016/j.redox.2014.06.004
141. Zhu YX, Jia HR, Gao G, Pan GY, Jiang YW, Li P, et al. Mitochondria-acting nanomicelles for destruction of cancer cells via excessive mitophagy/autophagy-driven lethal energy depletion and phototherapy. *Biomaterials* (2020) 232:119668. doi: 10.1016/j.biomaterials.2019.119668
142. Narendra DP, Jin SM, Tanaka A, Suen D-F, Gautier CA, Shen J, et al. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PloS Biol* (2010) 8:e1000298. doi: 10.1371/journal.pbio.1000298
143. Lahiri V, Hawkins WD, Klionsky DJ. Review Watch What You (Self-) Eat: Autophagic Mechanisms that Modulate Metabolism. *Cell Metab* (2019) 29:803–26. doi: 10.1016/j.cmet.2019.03.003
144. Fateye B, Li W, Wang C, Chen B. Combination of Phosphatidylinositol 3-Kinases Pathway Inhibitor and Photodynamic Therapy in Endothelial and Tumor Cells. *Photochem Photobiol* (2012) 88:1265–72. doi: 10.1111/j.1751-1097.2012.01160.x
145. Fateye B, Wan A, Yang X, Myers K, Chen B. Comparison between endothelial and tumor cells in the response to verteporfin-photodynamic therapy and a PI3K pathway inhibitor. *Photodiagn Photodyn Ther* (2015) 12:19–26. doi: 10.1016/j.pdpdt.2015.01.004
146. Niu T, Tian Y, Wang G, Guo G, Tong Y, Shi Y. Inhibition of ROS-NF-κB-dependent autophagy enhances Hypocrellin A united LED red light-induced apoptosis in squamous carcinoma A431 cells. *Cell Signal* (2020) 69:109550. doi: 10.1016/j.cellsig.2020.109550
147. Chen Y, Yin H, Tao Y, Zhong S, Yu H, Li J, et al. Antitumor effects and mechanisms of pyropheophorbide–α methyl ester–mediated photodynamic therapy on the human osteosarcoma cell line MG–63. *Int J Mol Med* (2020) 45:971–82. doi: 10.3892/ijmm.2020.4494
148. Gremke N, Polo P, Dort A, Schneikert J, Elmshäuser S, Brehm C, et al. mTOR-mediated cancer drug resistance suppresses autophagy and generates a druggable metabolic vulnerability. *Nat Commun* (2020) 11:4684. doi: 10.1038/s41467-020-18504-7

149. Duan X, Chen B, Cui Y, Zhou L, Wu C, Yang Z, et al. Ready player one? Autophagy shapes resistance to photodynamic therapy in cancers. *Apoptosis* (2018) 23:587–606. doi: 10.1007/s10495-018-1489-0
150. Yoshimori T, Yamamoto A, Moriyama Y, Futai M, Tashiro Y. Bafilomycin A1, a specific inhibitor of vacuolar-type H+-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *J Biol Chem* (1991) 266:17707–12.
151. Yamamoto A, Tagawa Y, Yoshimori T, Moriyama Y, Masaki R, Tashiro Y. Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. *Cell Struct Funct* (1998) 23:33–42. doi: 10.1247/csf.23.33
152. Klionsky DJ, Elazar Z, Seglen PO, Rubinsztein DC. Does bafilomycin A1 block the fusion of autophagosomes with lysosomes? *Autophagy* (2008) 4:849–50. doi: 10.4161/auto.6845
153. Tsubone TM, Rocha CS, Tonolli PN, Watanabe II –S, Stolf BS, Baptista MS, et al. *In vitro* Autophagy Modulation with Chloroquine: Some Lessons to Learn. *Adv Biochem Biotechnol* (2020) 5:1098. doi: 10.29011/2574-7258.001098
154. Liu T-J, Koul D, LaFortune T, Tiao N, Shen RJ, Maira S-M, et al. NVP-BEZ235, a novel dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor, elicits multifaceted antitumor activities in human gliomas. *Mol Cancer Ther* (2009) 8:2204–10. doi: 10.1158/1535-7163.MCT-09-0160
155. Lange C, Lehmann C, Mahler M, Bednarski PJ. Comparison of Cellular Death Pathways after mTHPC-mediated Photodynamic Therapy (PDT) in Five Human Cancer Cell Lines. *Cancers (Basel)* (2019) 11:702. doi: 10.3390/cancers11050702
156. Blommaart EFC, Krause U, Schellens JPM, Vreeling-Sindelarova H, Meijer AJ, Vreeling-Sindelárová H, et al. The Phosphatidylinositol 3-Kinase Inhibitors Wortmannin and LY294002 Inhibit Autophagy in Isolated Rat Hepatocytes. *Eur J Biochem* (1997) 243:240–6. doi: 10.1111/j.1432-1033.1997.0240a.x
157. Mauvezin C, Nagy P, Juhász G, Neufeld TP. Autophagosome–lysosome fusion is independent of V-ATPase-mediated acidification. *Nat Commun* (2015) 6:7007. doi: 10.1038/ncomms8007
158. Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* (2008) 4:151–75 doi: 10.4161/auto.5338
159. Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* (2012) 8:445–544. doi: 10.4161/auto.19496
160. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* (2016) 12:1–222. doi: 10.1080/15548627.2015.1100356
161. Domagala A, Stachura J, Gabrysiak M, Muchowicz A, Zagozdzon R, Golab J, et al. Inhibition of autophagy sensitizes cancer cells to Photofrin-based photodynamic therapy. *BMC Cancer* (2018) 18:210. doi: 10.1186/s12885-018-4126-y
162. Luo S, Xing D, Wei Y, Chen Q. Inhibitive effects of photofrin on cellular autophagy. *J Cell Physiol* (2010) 224:414–22. doi: 10.1002/jcp.22137
163. Ouyang G, Xiong L, Liu Z, Lam B, Bui B, Ma L, et al. Inhibition of autophagy potentiates the apoptosis-inducing effects of photodynamic therapy on human colon cancer cells. *Photodiagn Photodyn Ther* (2018) 21:396–403. doi: 10.1016/j.pdpdt.2018.01.010
164. Bretin L, Pinon A, Bouramtane S, Ouk C, Richard L, Perrin M, et al. Photodynamic Therapy Activity of New Human Colorectal Cancer. *Cancers (Basel)* (2019) 11:1–27. doi: 10.3390/cancers11101474
165. Andrzejak M, Price M, Kessel DH. Apoptotic and autophagic responses to photodynamic therapy in 1c1c7 murine hepatoma cells. *Autophagy* (2011) 7:979–84. doi: 10.4161/auto.7.9.15865
166. Wang H, Li L, Wang P, Wang X, Zhang K, Liu Q. Comparison of photodynamic treatment produced cell damage between human breast cancer cell MCF-7 and its multidrug resistance cell. *Photodiagn Photodyn Ther* (2016) 16:1–8. doi: 10.1016/j.pdpdt.2016.07.004
167. Feng X, Shi Y, Xie L, Zhang K, Wang X, Liu Q, et al. 2-deoxy-D-glucose augments photodynamic therapy induced mitochondrial caspase-independent apoptosis and energy-mediated autophagy. *Lasers Surg Med* (2019) 51:352–62. doi: 10.1002/lsm.23020
168. Huang Q, Ou YS, Tao Y, Yin H, Tu PH. Apoptosis and autophagy induced by pyropheophorbide-alpha methyl ester-mediated photodynamic therapy in human osteosarcoma MG-63 cells. *Apoptosis* (2016) 21:1–12. doi: 10.1007/s10495-016-1243-4
169. Kessel DH, Price M, Reiners JJ. ATG7 deficiency suppresses apoptosis and cell death induced by lysosomal photodamage. *Autophagy* (2012) 8:1333–41. doi: 10.4161/auto.20792
170. Mohammadalipour Z, Rahmati M, Khataee A, Moosavi MA. Differential effects of N-TiO2 nanoparticle and its photo-activated form on autophagy and necroptosis in human melanoma A375 cells. *J Cell Physiol* (2020) 235 (11):1–14. doi: 10.1002/jcp.29479
171. Ahn MY, Yoon HE, Kwon SM, Lee J, Min SK, Kim YC, et al. Synthesized Pheophorbide a-mediated photodynamic therapy induced apoptosis and autophagy in human oral squamous carcinoma cells. *J Oral Pathol Med* (2013) 42:17–25. doi: 10.1111/j.1600-0714.2012.01187.x
172. Miki Y, Akimoto J, Moritake K, Hironaka C, Fujiwara Y. Photodynamic therapy using talaporfin sodium induces concentration-dependent programmed necroptosis in human glioblastoma T98G cells. *Lasers Med Sci* (2015) 30:1739–45. doi: 10.1007/s10103-015-1783-9
173. Sun M, Zhou C, Zeng H, Puebla-Osorio N, Damiani E, Chen J, et al. Hiporfin-mediated photodynamic therapy in preclinical treatment of osteosarcoma. *Photochem Photobiol* (2015) 91:533–44. doi: 10.1111/php.12424
174. Soriano J, Mora-Espí I, Alea-Reyes ME, Pérez-García L, Barrios L, Ibáñez E, et al. Cell death mechanisms in Tumoral and Non-Tumoral human cell lines triggered by photodynamic treatments: Apoptosis, necrosis and parthanatos. *Sci Rep* (2017) 7:1–13. doi: 10.1038/srep41340
175. Shen S, Kepp O, Kroemer G. The end of autophagic cell death? *Autophagy* (2012) 8:1–3. doi: 10.4161/auto.8.1.16618
176. Galluzzi L, Vitale I, Aaronson SA, Abrams JM, Adam D, Agostinis P, et al. Molecular mechanisms of cell death: Recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death Differ* (2018) 25:486–541. doi: 10.1038/s41418-017-0012-4
177. Wang F, Wu H, Fan M, Yu R, Zhang Y, Liu J, et al. Sodium butyrate inhibits migration and induces AMPK-mTOR pathway-dependent autophagy and ROS-mediated apoptosis via the miR-139-5p/Bmi-1 axis in human bladder cancer cells. *FASEB J* (2020) 34:4266–82. doi: 10.1096/fj.201902626R
178. Wang J, Xing Y, Wang Y, He Y, Wang L, Peng S, et al. A novel BMI-1 inhibitor QW24 for the treatment of stem-like colorectal cancer. *J Exp Clin Cancer Res* (2019) 38:1–14. doi: 10.1186/s13046-019-1392-8
179. Dey A, Mustafi SB, Saha S, Kumar Dhar Dwivedi S, Mukherjee P, Bhattacharya R. Inhibition of BMI1 induces autophagy-mediated necroptosis. *Autophagy* (2016) 12:659–70. doi: 10.1080/15548627.2016.1147670
180. Huang L, Lin H, Chen Q, Yu L, Bai D. MPPa-PDT suppresses breast tumor migration/invasion by inhibiting Akt-NF-κB-dependent MMP-9 expression via ROS. *BMC Cancer* (2019) 19:1–11. doi: 10.1186/s12885-019-6374-x
181. Kessel D, Reiners JJ. Enhanced Efficacy of Photodynamic Therapy via a Sequential Targeting Protocol. *Photochem Photobiol* (2014) 4:889–95. doi: 10.1111/php.12270
182. Kessel D. Photodynamic therapy: Promotion of efficacy by a sequential protocol. *J Porphyr Phthalocyanines* (2016) 20:302–6. doi: 10.1142/S1088424616500073
183. Serra V, Markman B, Scaltriti M, Eichhorn PJA, Valero V, Guzman M, et al. NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations. *Cancer Res* (2008) 68:8022–30. doi: 10.1158/0008-5472.CAN-08-1385
184. Kharkwal GB, Sharma SK, Huang Y-Y, Dai T, Hamblin MR. Photodynamic therapy for infections: Clinical applications. *Lasers Surg Med* (2011) 43:755–67. doi: 10.1002/lsm.21080
185. Sharma SK, Mroz P, Dai T, Huang YY, Denis TGS, Hamblin MR. Photodynamic therapy for cancer and for infections: What is the difference? *Isr J Chem* (2012) 52:691–705. doi: 10.1002/ijch.201100062
186. Korbelik M. Photodynamic Therapy-Generated Cancer Vaccines. In: Chapter 12. Antimicrobial Photodynamic Inactivation and Photodynamic Therapy for Infections. *Methods Mol Biol* (2020) 147–53. doi: 10.1007/978-1-60761-697-9_11

187. Kabingu E, Oseroff AR, Wilding GE, Gollnick SO. Enhanced Systemic Immune Reactivity to a Basal Cell Carcinoma Associated Antigen Following Photodynamic Therapy. *Clin Cancer Res* (2009) 15:4460–6. doi: 10.1158/1078-0432.CCR-09-0400
188. Gollnick SO, Brackett CM. Enhancement of anti-tumor immunity by photodynamic therapy. *Immunol Res* (2010) 46:216–26. doi: 10.1007/s12026-009-8119-4
189. National Institutes of Health. Clinical Trial. Gov: A service of the U.S. National Institutes of Health. (2016).
190. Perera E, Gnaneswaran N, Staines C, Win AK, Sinclair R. Incidence and prevalence of non-melanoma skin cancer in Australia: A systematic review. *Australas J Dermatol* (2015) 56:258–67. doi: 10.1111/ajd.12282
191. Lv R, Sun Q. A Network Meta-Analysis of Non-Melanoma Skin Cancer (NMSC) Treatments: Efficacy and Safety Assessment. *J Cell Biochem* (2017) 118:3686–95. doi: 10.1002/jcb.26015
192. Marcil I, Stern RS. Risk of developing a subsequent nonmelanoma skin cancer in patients with a history of nonmelanoma skin cancer: A critical review of the literature and meta-analysis. *Arch Dermatol* (2000) 136:1524–30. doi: 10.1001/archderm.136.12.1524
193. Lara F, Garbers LEF de M, Santamaría JR. Recurrence rate of basal cell carcinoma with positive histopathological margins and related risk factors. *Bras Dermatol* (2017) 92:58–62. doi: 10.1590/abd1806-4841.20174867
194. Wetzig T, Maschke J, Kendler M, Simon JC. Treatment of basal cell carcinoma. *JDDG - J Ger Soc Dermatol* (2009) 7:1075–83. doi: 10.1111/j.1610-0387.2009.07097.x
195. Patel RV, Frankel A, Goldenberg G. An update on nonmelanoma skin cancer. *J Clin Aesthet Dermatol* (2011) 4:20–7.
196. Basset-Seguin N, Ibbotson SH, Emtestam L, Tarstedt M, Morton C, Maroti M, et al. Topical methyl aminolaevulinate photodynamic therapy versus cryotherapy for superficial basal cell carcinoma: a 5 year randomized trial. *Eur J Dermatol* (2008) 18(5):547–53. doi: 10.1684/ejd.2008.0472
197. Morton CA, Dominicus R, Radny P, Dirschka T, Hauschild A, Reinhold U, et al. A randomized, multinational, noninferiority, phase III trial to evaluate the safety and efficacy of BF-200 aminolaevulinic acid gel vs. methyl aminolaevulinate cream in the treatment of nonaggressive basal cell carcinoma with photodynamic therapy. *Br J Dermatol* (2018) 179:309–19. doi: 10.1111/bjd.16441
198. Nguyen NTA, Ringash J. Head and Neck Cancer Survivorship Care: A Review of the Current Guidelines and Remaining Unmet Needs. *Curr Treat Options Oncol* (2018) 19(8):44–64. doi: 10.1007/s11864-018-0554-9
199. Lou PJ, Jäger HR, Jones L, Theodossy T, Bown SG, Hopper C. Interstitial photodynamic therapy as salvage treatment for recurrent head and neck cancer. *Br J Cancer* (2004) 91:441–6. doi: 10.1038/sj.bjc.6601993
200. Li W, Yang J, Luo L, Jiang M, Qin B, Yin H, et al. Targeting photodynamic and photothermal therapy to the endoplasmic reticulum enhances immunogenic cancer cell death. *Nat Commun* (2019) 10(1):3349. doi: 10.1038/s41467-019-11269-8
201. Civantos F. Photodynamic therapy for head and neck lesions in the subtropics. *JNCCN J Natl Compr Cancer Netw* (2012) 10:65–8. doi: 10.6004/jnccn.2012.0179
202. Biel MA. Photodynamic therapy treatment of early oral and laryngeal cancers. *Photochem Photobiol* (2007) 83:1063–8. doi: 10.1111/j.1751-1097.2007.00153.x
203. Rigual NR, Thankappan K, Cooper M, Sullivan MA, Dougherty T, Popat SR, et al. Photodynamic therapy for head and neck dysplasia and cancer. *Arch Otolaryngol - Head Neck Surg* (2009) 135:784–8. doi: 10.1001/archoto.2009.98
204. Hillemanns P, Garcia F, Petry KU, Dvorak V, Sadovsky O, Iversen OE, et al. A randomized study of hexaminolevulinate photodynamic therapy in patients with cervical intraepithelial neoplasia 1/2. *Am J Obstet Gynecol* (2015) 212:465.e1–7. doi: 10.1016/j.ajog.2014.10.1107
205. Kato H. Our experience with photodynamic diagnosis and photodynamic therapy for lung cancer. *JNCCN J Natl Compr Cancer Netw* (2012) 10:6–7. doi: 10.6004/jnccn.2012.0170
206. Wisnivesky JP, Yung RCW, Mathur PN, Zulueta JJ. Diagnosis and treatment of bronchial intraepithelial neoplasia and early lung cancer of the central airways: Diagnosis and management of lung cancer, 3rd ed: American college of chest physicians evidence-based clinical practice guidelines. *Chest* (2013) 143:e263S–77S. doi: 10.1378/chest.12-2358
207. Furuse K, Fukuoka M, Kato H, Horai T, Kubota K, Kodama N, et al. A prospective phase II study on photodynamic therapy with photofrin II for centrally located early-stage lung cancer. *J Clin Oncol* (1993) 11:1852–7. doi: 10.1200/JCO.1993.11.10.1852
208. Kato H, Furukawa K, Sato M, Okunaka T, Kusunoki Y, Kawahara M, et al. Phase II clinical study of photodynamic therapy using mono-L-aspartyl chlorin e6 and diode laser for early superficial squamous cell carcinoma of the lung. *Lung Cancer* (2003) 42:103–11. doi: 10.1016/S0169-5002(03)00242-3
209. Martins WK, Baptista MS. Autophagy Modulation for Organelle-Targeting Autophagy Modulation for Organelle-Targeting Therapy. In: *Autophagy in Current Trends in Cellular Physiology and Pathology* (2016) p. 350–90. doi: 10.5772/32009
210. Dos Santos AF, De Almeida DRQ, Terra LF, Baptista MS, Labriola L. Photodynamic therapy in cancer treatment - an update review. *J Cancer Metastasis Treat* (2019) 5(25):1–20. doi: 10.20517/2394-4722.2018.83

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Martins, Belotto, Silva, Grasso, Suriani, Lavor, Itri, Baptista and Tsubone. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 21 January 2021
doi: 10.3389/fcell.2020.596655

Metformin Resensitizes Sorafenib-Resistant HCC Cells Through AMPK-Dependent Autophagy Activation

Hong-Yue Lai[1,2†], Hsin-Hwa Tsai[1,3†], Chia-Jui Yen[4,5], Liang-Yi Hung[1,5,6], Ching-Chieh Yang[7,8], Chung-Han Ho[2,9], Hsin-Yin Liang[1,10], Feng-Wei Chen[11], Chien-Feng Li[2,3,12,13,14] and Ju-Ming Wang[1,6,10,15*]*

[1] Department of Biotechnology and Bioindustry Sciences, College of Bioscience and Biotechnology, National Cheng Kung University, Tainan, Taiwan, [2] Department of Medical Research, Chi Mei Medical Center, Tainan, Taiwan, [3] Department of Pathology, Chi Mei Medical Center, Tainan, Taiwan, [4] Department of Oncology, National Cheng Kung University Hospital, Tainan, Taiwan, [5] College of Medicine, National Cheng Kung University, Taipei, Taiwan, [6] College of Medicine, Graduate Institute of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, [7] Department of Radiation Oncology, Chi-Mei Medical Center, Tainan, Taiwan, [8] Department of Pharmacy, Chia-Nan University of Pharmacy and Science, Tainan, Taiwan, [9] Department of Hospital and Health Care Administration, Chia Nan University of Pharmacy and Science, Tainan, Taiwan, [10] International Center for Wound Repair and Regeneration, National Cheng Kung University, Tainan, Taiwan, [11] Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan, Taiwan, [12] National Institute of Cancer Research, National Health Research Institute, Tainan, Taiwan, [13] Institute of Clinical Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, [14] Department of Biotechnology, Southern Taiwan University of Science and Technology, Tainan, Taiwan, [15] College of Medical Science and Technology, Graduate Institute of Medical Sciences, Taipei Medical University, Taipei, Taiwan

OPEN ACCESS

Edited by:
Daniel Hector Grasso, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

Reviewed by:
Cristián Favre, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
Keith R. Laderoute, SRI International, United States

***Correspondence:**
Ju-Ming Wang
yumingw@mail.ncku.edu.tw
Chien-Feng Li
cfli@mail.chimei.org.tw

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: 20 August 2020
Accepted: 22 December 2020
Published: 21 January 2021

Citation:
Lai H-Y, Tsai H-H, Yen C-J, Hung L-Y, Yang C-C, Ho C-H, Liang H-Y, Chen F-W, Li C-F and Wang J-M (2021) Metformin Resensitizes Sorafenib-Resistant HCC Cells Through AMPK-Dependent Autophagy Activation. Front. Cell Dev. Biol. 8:596655. doi: 10.3389/fcell.2020.596655

Despite the activation of autophagy may enable residual cancer cells to survive and allow tumor relapse, excessive activation of autophagy may eventually lead to cell death. However, the details of the association of autophagy with primary resistance in hepatocellular carcinoma (HCC) remain less clear. In this study, cohort analysis revealed that HCC patients receiving sorafenib with HBV had higher mortality risk. We found that high epidermal growth factor receptor (EGFR) expression and activity may be linked to HBV-induced sorafenib resistance. We further found that the resistance of EGFR-overexpressed liver cancer cells to sorafenib is associated with low activity of AMP-activated protein kinase (AMPK) and CCAAT/enhancer binding protein delta (CEBPD) as well as insufficient autophagic activation. In response to metformin, the AMPK/cAMP-response element binding protein (CREB) pathway contributes to CEBPD activation, which promotes autophagic cell death. Moreover, treatment with metformin can increase sorafenib sensitivity through AMPK activation in EGFR-overexpressed liver cancer cells. This study suggests that AMPK/CEBPD-activated autophagy could be a potent strategy for improving the efficacy of sorafenib in HCC patients.

Keywords: sorafenib, metformin, autophagy, AMPK, CEBPD

INTRODUCTION

Sorafenib is a multi-kinase inhibitor that mainly targets Raf kinases and receptor tyrosine kinases, including vascular endothelial growth factor receptor (VEGFR)-2/3, platelet-derived growth factor receptor (PDGFR)-β, FMS-like tyrosine kinase 3 (Flt3), and c-Kit (CD117) (Cervello et al., 2012), which are involved in tumor angiogenesis and progression. However, the overall outcomes for

patients with advanced hepatocellular carcinoma (HCC) are discouraging and distinct tumor cells exhibit diverse degrees of sensitivity to sorafenib. Therefore, a precise understanding of the mechanism of resistance is critical to develop personalized medicine strategies for HCC patients.

Autophagy is a highly conserved intracellular degradation process that can be enhanced when cancer cells face environmental stresses such as nutritional deficiency and even chemotherapy. Autophagy induced by hepatitis B virus (HBV)/hepatitis C virus (HCV) has been suggested to support viral replication and contributes to HCC progression (Wang et al., 2014; Wu et al., 2014; Khan et al., 2018). Epidermal growth factor receptor (EGFR) is overexpressed and activated in more than half of HCC patients (Buckley et al., 2008). A combination of EGFR inhibitor and sorafenib was assessed as a rational therapeutic strategy for HCC (Zhu et al., 2017), but the preclinical results were far from satisfactory. Some studies showed that autophagy induced by EGFR inhibitors is cytoprotective, and the combination of EGFR inhibitors with autophagy inhibitors might be beneficial (Wang et al., 2019). Despite autophagy is involved in a survival mechanism, excessive activation of autophagy could eventually lead to cell death (Liu and Levine, 2015). Autophagy is also responsive to sorafenib stress and strengthens the sorafenib-induced death of cancer cells (Park et al., 2010). Therefore, the complex role of autophagy should be clarified, which may be important to precisely regulate the levels of autophagy to control HCC.

CCAAT/enhancer binding protein delta (CEBPD) is a transcription factor that responds to various external stimuli, including the proinflammatory cytokines IL-1β and TNFα (Chang et al., 2012), stress (O'Rourke et al., 1997), growth factors (Wang et al., 2005), and anti-cancer chemotherapy drugs (Li et al., 2015; Chu et al., 2017; Tsai et al., 2017). CEBPD is thought to be a potent tumor suppressor, and its expression is downregulated in several cancers, including breast cancer (Sivko and DeWille, 2004), leukemia (Agrawal et al., 2007), cervical cancer (Pan et al., 2010), and hepatocellular carcinoma. We previously demonstrated that epigenetic regulation contributes to CEBPD inactivation in cancers (Ko et al., 2008) and that strong CEBPD activation can strengthen the death of cancer cells via eliminating epigenetic control (Li et al., 2015). However, we also found that inhibition of the EGFR/STAT3/CEBPD axis reverses cisplatin resistance in bladder cancer (Wang et al., 2017). Therefore, the different and sometime paradoxical function of CEBPD appears to be dependent on cell type-specific contexts.

Metformin (dimethylbiguanide) is a current first-line pharmacological treatment for type 2 diabetes. Some studies have further demonstrated that metformin can induce cell arrest and promote cell death (Chen et al., 2013). Metformin can activate autophagy by inhibiting mammalian target of rapamycin (mTOR) directly or indirectly in an AMP-activated protein kinase (AMPK)-dependent manner (Kim and He, 2013; Pernicova and Korbonits, 2014). It has also been suggested that metformin should be applied for therapy for other cancers in addition to HCC, including melanoma and lymphoma, via autophagic activation (Tomic et al., 2011; Shi et al., 2012). However, the molecular details of metformin in overcoming the primary resistance of liver cancer cells to sorafenib remains an open question.

Abbreviations: AMPK, AMP-activated protein kinase; CEBPD, CCAAT/enhancer binding protein delta; EGFR, epidermal growth factor receptor; ERK, extracellular-signal-regulated kinase; HBV, hepatitis B virus; HBx, HBV-encoded X protein; HCC, hepatocellular carcinoma; LC3B, light chain 3 beta.

MATERIALS AND METHODS

Clinical Data Analysis

Our nationwide cohort analysis used the Taiwan Cancer Registry (TCR) and National Health Insurance Research Database (NHIRD) to identify diagnosis of HCC and sorafenib prescription (Lu et al., 2017; Chan et al., 2019). The TCR database captures 97% of the cancer cases in Taiwan and also represented a perfect data quality comparing to other well-established cancer registries (Bray and Parkin, 2009; Chiang et al., 2016). To ensure patient privacy, all personal identifying information was removed prior to analysis. This study was approved by the Institutional Review Board of Chi-Mei Medical Center in Taiwan (IRB: 10702-E04).

The International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) code 155.0 were used to identify patients diagnosed with HCC between 2012 and 2015 from the TCR database. Information on HBV or HCV infection were obtained for the period from 12 months before until 12 months after HCC diagnosis based on ICD-9-CM diagnosis codes: HBV (070.20, 070.22, 070.30, 070.32) and HCV (070.41, 070.44, 070.51, 070.70, 070.71). Patients with a previous cancer history, a lack of clear demographic and tumor information or aged <18 years were excluded. Finally, a total of 6,628 HCC patients were enrolled in this analysis. For the usage of sorafenib, all patients were reimbursed without co-payment by NHI according to the criteria BCLC advanced stage that were not amenable to either surgical resection or locoregional therapy and Child–Pugh class A liver functional reserve. The prescription of sorafenib is 800 mg (200 mg/tablet) for 2 months. The application needed to be re-evaluated every 2 months for next term of sorafenib usage with imaging evidence showing no disease progression.

The categorical variables were presented as frequency with percentage, and the difference between patients with HBV diagnosed and those without was compared using Pearson's chi-square test. The 1-year mortality risk for HCC patients with different hepatitis B/C virus was estimated using Cox proportional regression analysis adjusted with age, gender, HCC diagnosed to start sorafenib, dosage of sorafenib, comorbidities, and additional therapy after sorafenib such as TACE, RFA, radiation, hepatectomy, and liver transplantations. The stratified analysis was also implemented to investigate the mortality risk among the different duration of sorafenib used. In addition, the estimation of different follow-up period mortality risk was considered. The predicted survival curves were plotted using the results of above Cox regression analysis with adjusted confounding factors. SAS 9.4 for Windows (SAS Institute, Inc., Cary, NC, USA) was used for all statistical analyses. All

statistical tests were 2-sided, and $p < 0.05$ was considered statistically significant.

Cell Culture

The human hepatocellular carcinoma cell lines Huh7 and Hep3B were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 units/ml penicillin at 37°C and 5% CO_2.

Lentiviral shRNA Knockdown

The virus was produced from Phoenix Ampho cells using Mirus Bio TransIT-2020 and cotransfected with various short hairpin RNA (shRNA) expression vectors in combination with pMD2.G and psPAX2 vectors and the pLKO.1-shRNA expression vectors. The short interfering RNA sequences targeting LacZ, CEBPD, and AMPK were subcloned into the lentiviral expression vector pLKO.1. The short interfering RNA sequences are as follows: shLacZ (shZ): 5′-CCGGTGTTCGCATTATCCGAA CCATCTCGAGATGGTTCGGATAATGCGAACATTTTT G-3′; shCEBPD (shD): 5′-CCGGGCCGACCTCTTCAACAG CAATCTCGAGATTGCTGTTGAAGAGGTCGGCTTTTT-3′; shAMPKα (shKα1): 5′-CCGGTGATTGATGATGAAGCC TTAACTCGAGTTAAGGCTTCATCATCAATCATTTTT-3′; shAMPKα (shKα2): 5′-CCGGCAACTTTACCTGGTTGATAA CCTCGAGGTTATCAACCAGGTAAAGTTGTTTT-3′. The expression vectors and shRNAs were obtained from the National RNAi Core Facility located at the Genomic Research Center of Institute of Molecular Biology, Academia Sinica, Taiwan.

Plasmid Transfection and Reporter Assays

Human CEBPD reporter was constructed in our lab (Wang et al., 2005). The reporter was transfected into Huh7 cells by Turbofect according to the manufacturer's suggestions. Transfectants were cultured in complete medium with or without treatment for 3 h. Luciferase activity was measured in the lysates of transfectants.

Cell Viability

Huh7 and Hep3B cells were seeded $5*10^3$ cells per well in 96-well plates. Cells were treated with various concentrations (0, 2.5, and 5 μM) of sorafenib for 48 h or with the combination of 2.5 μM sorafenib and 5 mM metformin for 48 h. The experimental cells were incubated with diluted MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] at 37°C for 3.5 h. The samples were then measured spectrophotometrically at 595 nm by an ELISA plate reader.

Flow Cytometry Analysis

Huh7 and Hep3B cells were treated with sorafenib for 48 h. Treated and control cells were harvested, washed twice and re-suspended in 500 μl of PBS plus Annexin V-FITC and PI in dark for 15 min at room temperature. The degree of apoptosis was determined as the percentage of cells positive for Annexin V-FITC/PI. For each sample, at least 1×10^4 cells were analyzed by FACScan cytometry (CellLab Quanta^{TM} SC, *Beckman Coulter*). The data were determined by three independent experiments.

Fluorescence Microscopy

The pEGFP-LC3 plasmid was a gift obtained from Dr. Tamotsu Yahsimori and Noboru Mizushima (Kabeya et al., 2000). Huh7 and Hep3B cells transfected with GFP-LC3B plasmid were grown on glass coverslips or treated with sorafenib (2.5 and 5 μM) for 6 h, and then examined under a fluorescence microscope. Images shown are representative of three independent experiments. The fold changes of the average numbers of puncta per positive cells were calculated with 50 individual cells.

Animal Studies

Male, 6-week-old NOD/SCID mice were obtained from the Laboratory Animal Center of National Cheng Kung University, Tainan, Taiwan. Hep3B cells (5×10^6) in 0.2 ml PBS were inoculated subcutaneously into the right flank of the mice. After 14 days, when macroscopic tumors (50–100 mm^3) had formed, animals were placed randomly into four groups ($n = 5$ per group) as follows: (1) the control group, which received identical volumes of vehicle; (2) the sorafenib treatment group, which was treated with sorafenib at doses of 15 mg/kg/day; (3) the metformin treatment group, which was treated with 250 mg/kg/day metformin; and (4) the combined treatment group, which was injected with sorafenib combined with metformin. Treatment was given to all groups intraperitoneally every day for 4 weeks. Animal weight and tumor dimensions were measured every 4 days with calipers, and tumor volumes were estimated using two-dimensional measurements of length and width and were calculated with the formula: $[l \times (w)^2] \times 0.52$, where l is length and w is width.

Statistical Analysis

All experiments were repeated at least 3 times, and data were analyzed for statistical significance by two-tailed unpaired Student's *t*-test using Prism 5 software. The data were expressed as the means ± SEM. Differences were considered statistically significant when indicated by asterisks.

RESULTS

HBV Is Associated With Sorafenib Resistance in HCC Cells

HBV and HCV are major risk factors for HCC and have been associated with therapeutic efficacy. To check the clinical relevance of HBV/HCV with sorafenib resistance in patients with HCC, cohort analysis was performed to identify HCC patients receiving sorafenib with ($n = 3{,}389$) or without HBV/HCV ($n = 2{,}113$) (**Table 1**). After adjusted to potential confounding factors, patients with HBV/HCV increased 10% risk of overall 1-year mortality compared with those without HBV/HCV (**Table 2**). In addition, patients with HBV/HCV had higher mortality risk at 6–12 months follow-up period than those without HBV/HCV (**Table 3**), and the estimated survival probability from the hazard function after adjusted to age, gender, HCC diagnosed to start sorafenib, dosage of sorafenib, comorbidities, and additional therapy after sorafenib was plotted as **Figure 1A**. Interestingly, previous study indicated that sorafenib improved overall survival among patients with HCC who were HCV positive but HBV

TABLE 1 | Clinical information of HCC patients treated with sorafenib with or without HBV/HCV.

Characteristic	HBV diagnosed (with HBV alone or both HBV/HCV)		No HBV and HCV diagnosed		*P*-value*
	N	%	*N*	%	
Overall patients	3,389	61.6	2,113	38.4	
Age groups					
<35	114	3.36	33	1.56	<0.0001
35–50	769	22.69	195	9.23	
50–65	1,684	49.69	813	38.48	
>65	822	24.25	1,072	50.73	
Gender, male	2,917	86.07	1,661	78.61	<0.0001
HCC diagnosed to start sorafenib, months					
<3	1,564	46.15	1,181	55.89	<0.0001
3–6	476	14.05	201	9.51	
6–12	521	15.37	253	11.97	
>12	828	24.43	478	22.62	
Duration of sorafenib used, months					
<2	1,703	50.25	1138	53.86	0.0787
2–4	692	20.42	400	18.93	
4–6	301	8.88	176	8.33	
>6	693	20.45	399	18.88	
Dosage of sorafenib					
<240	173	5.1	152	7.19	<0.0001
240–480	1,136	33.52	813	38.48	
480–720	259	7.64	168	7.95	
>720	1,821	53.73	980	46.38	
Comorbidities					
Alcoholic liver disease	197	5.81	168	7.95	0.0019
Liver cirrhosis	1,184	34.94	547	25.89	<0.0001
Liver decompensation	197	5.81	125	5.92	0.8744
Diabetes mellitus	922	27.21	873	41.32	<0.0001

**P-value was estimated using Pearson's chi-square test.*

TABLE 2 | Risk of 1-year mortality in HCC patients receiving sorafenib with or without HBV/HCV.

Characteristic	Adjusted HR[a] (95% CI)				
	Overall	Duration of sorafenib used, months			
		<2	2–4	4–6	>6
HBV diagnosed (with HBV alone or both HBV/HCV)	1.10 (1.03–1.19)*	1.04 (0.95–1.14)	1.21 (1.04–1.41)*	1.29 (0.99–1.68)	1.27 (0.92–1.75)
No HBV and HCV diagnosed	Ref.	Ref.	Ref.	Ref.	Ref.

HR, hazard ratio; CI, confidence interval.
[a]Adjusted for age groups, gender, HCC diagnosed to start sorafenib, dosage of sorafenib, comorbidities, and additional therapy after sorafenib.
**P < 0.05.*

negative (Jackson et al., 2017), suggesting that HBV might be the major cause of sorafenib resistance. To dissect the presence of HBV in sorafenib resistance, two human HCC cell lines, Huh7 without HBV and Hep3B with an integrated HBV genome, were treated with different concentrations (2.5 and 5 μM) of sorafenib to address this issue. A cell viability assay revealed that Huh7 cells were more sensitive than Hep3B cells to sorafenib (**Figure 1B**). Furthermore, a cell death assay revealed that sorafenib significantly induced apoptotic cell death in Huh7 cells compared to Hep3B cells (**Figure 1C**), suggesting that Hep3B cells are intrinsically more resistant than Huh7 cells to sorafenib.

High EGFR Activity and Low AMPK Activity Determine the Primary Resistance of Hep3B Cells to Sorafenib

To check the efficacy of sorafenib, the Raf downstream effector extracellular-signal-regulated kinase 1/2 (ERK1/2), a potential

TABLE 3 | Risk of follow-up period mortality in HCC patients receiving sorafenib with or without HBV/HCV.

Characteristic	Adjusted HR[a] (95% CI)				
	Overall	Follow-up period, months			
		<2	2–4	4–6	6–12
HBV diagnosed (with HBV alone or both HBV/HCV)	1.10 (1.03–1.19)*	1.03 (0.90–1.19)	1.02 (0.89–1.17)	1.06 (0.90–1.25)	1.30 (1.14–1.49)*
No HBV and HCV diagnosed	Ref.	Ref.	Ref.	Ref.	Ref.

HR, hazard ratio; CI, confidence interval.
[a]Adjusted for age groups, gender, HCC diagnosed to start sorafenib, dosage of sorafenib, comorbidities, and additional therapy after sorafenib.
**P < 0.05.*

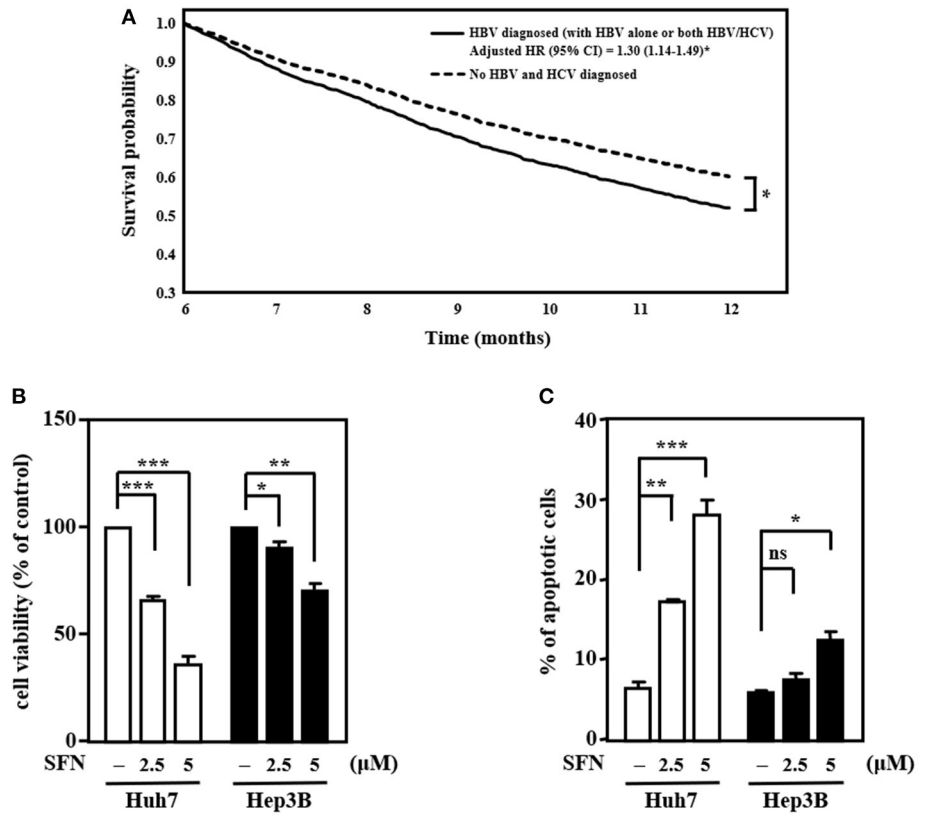


FIGURE 1 | HCC cells with HBV are more resistant than those without HBV to the anti-cancer effects of sorafenib. **(A)** The survival curves for more than 6 months follow-up period. **(B)** Huh7 and Hep3B cells were treated with sorafenib (SFN) at the indicated concentrations for 48 h. The cell viability of the experimental cells was measured by MTT assays after 48 h of sorafenib treatment at the indicated concentrations. **(C)** Huh7 and Hep3B cells were treated with sorafenib at the indicated concentrations for 48 h. Experimental cells were collected after 48 h of sorafenib treatment at the indicated concentrations, stained with Annexin-V/PI, and analyzed by flow cytometry. The data are shown as the mean ± SD. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 by Student's *t*-test.

biomarker for sorafenib response, was examined in Huh7 (*HBV*-negative) and Hep3B (derived from *HBV*-infected liver) cells. The results showed that, in contrast to that in Huh7 cells, the activity of ERK1/2 (phosphorylated ERK1/2, pERK1/2) was sustainedly activated in Hep3B cells, and there was no further effect following sorafenib treatment (**Figure 2A**). HBV-encoded X protein (HBx) has been suggested to increase EGFR expression by inhibiting miR129-5p function (Ochi et al., 2020). Here, we found that upregulation of EGFR in *HBV*-infected liver tissues compared with healthy liver tissues through analysis of the public dataset GSE83148 (**Supplementary Figure 1A**). To further dissect whether EGFR contributes to sorafenib resistance in Hep3B cells, the activity of EGFR (phosphorylated EGFR, pEGFR) was examined. Western blot analyses revealed that the basal levels of EGFR and pEGFR were higher in Hep3B cells than in Huh7 cells (**Figure 2B**) and that the EGFR inhibitor gefitinib

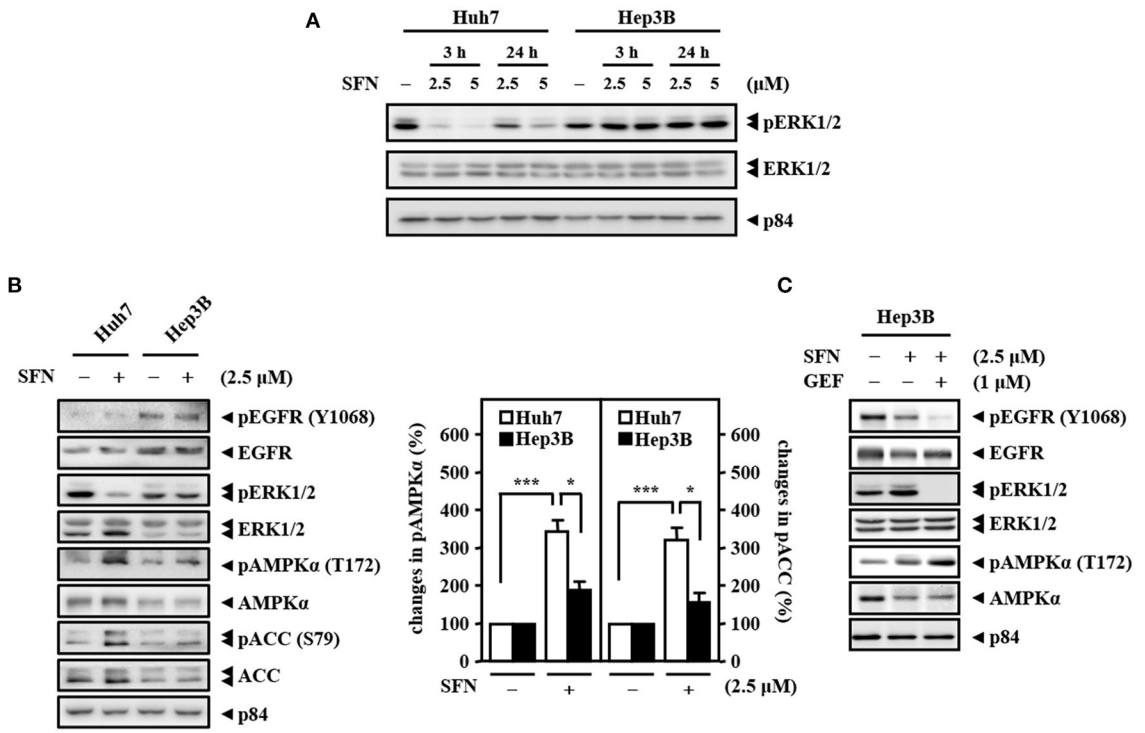


FIGURE 2 | High activity of EGFR and low activity of AMPK determine the primary resistance of Hep3B cells to sorafenib. **(A)** Huh7 and Hep3B cells were treated with sorafenib at the indicated concentrations for 3 and 24 h. Whole cell lysates were harvested for Western blot analyses. **(B)** Two types of liver cancer cells (Huh7 and Hep3B) were treated with or without sorafenib (2.5 μM) for 24 h and harvested for Western blot analyses. The quantitative analyses of phosphorylated AMPK and ACC were shown in the graphs. **(C)** Hep3B cells were treated with sorafenib (2.5 μM) alone or with the combination of sorafenib and the EGFR inhibitor gefitinib (GEF; 1 μM) for 24 h. Whole cell lysates were harvested for Western blot analyses. The data are shown as the mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by Student's *t*-test.

increased the efficacy of sorafenib by reducing the level of the phosphorylated ERK1/2 protein in Hep3B cells (**Figure 2C**). The above results suggest that the EGFR/ERK pathway may be linked to HBV-induced sorafenib resistance. Previous studies have indicated that ERK1/2 can promote the uncoupling of liver kinase B1 (LKB1) and AMPK to confer anti-apoptotic effects (Esteve-Puig et al., 2009). Here, we showed that the levels of phosphorylated AMPK and its downstream target acetyl-CoA carboxylase (ACC) were lower in Hep3B cells than in Huh7 cells under sorafenib treatment (**Figure 2B**) and that the EGFR inhibitor gefitinib strengthened sorafenib-induced AMPK phosphorylation in Hep3B cells (**Figure 2C**). Collectively, these results imply that the EGFR/ERK-induced reduction in AMPK phosphorylation plays a functional role in hepatocarcinoma resistance to sorafenib.

Resistance of Hep3B Cells to Sorafenib Is Associated With Lower Autophagic Responsiveness

Accumulated results have suggested that AMPK is an upstream activator of autophagy. Meanwhile, autophagy can serve as a tumor suppressor, and its deficiency leads to HCC (Liang et al., 2006; Takamura et al., 2011). However, whether AMPK and autophagy are involved in primary resistance to sorafenib in liver cancer cells remains unknown. Our results demonstrated that the levels of AMPK phosphorylation and the LC3B-II/LC3B-I ratio were significantly higher in Huh7 cells than in Hep3B cells upon sorafenib treatment (**Figures 3A,B**). In addition, sorafenib significantly increased the number of LC3B puncta in GFP-LC3B/Huh7 cells, but the number of LC3B puncta was marginally higher in GFP-LC3B/Hep3B cells (**Figure 3C**). The results suggest that the sorafenib resistance in liver cancer cells with EGFR overexpression is associated with insufficient autophagic activation.

CEBPD Is Involved in Sorafenib-Induced Autophagic Cell Death

Our previous results showed that CEBPD expression is responsive to clinical anti-cancer drugs in liver cancer cells (Li et al., 2015). Here, we found that co-downregulation of CEBPD and LC3B in $EGFR^{high}$ *HBV*-infected liver tissues compared with $EGFR^{low}$ *HBV*-infected liver tissues through analysis of the public dataset GSE83148 (**Supplementary Figure 1B**). We further validated the liver specimens from the HBx transgenic mice by immunofluorescence. Consistently, we found that CEBPD and LC3B expressions were lower in tumors (T) compared with adjacent non-tumor (N) tissues (**Supplementary Figure 2**). Interestingly, we found that

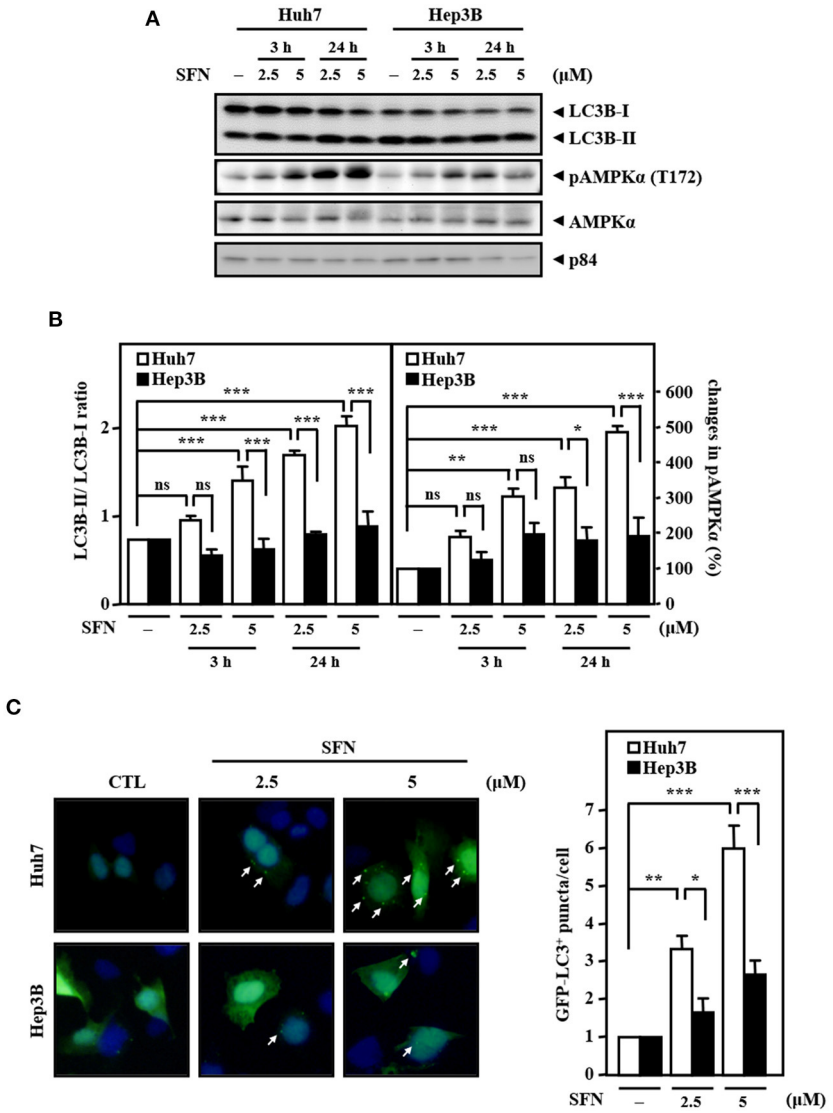


FIGURE 3 | Resistance of Hep3B cells to sorafenib is associated with lower autophagic responsiveness. **(A)** Huh7 and Hep3B cells were treated with sorafenib at the indicated concentrations for 3 and 24 h. Whole cell lysates were harvested for Western blot analyses. **(B)** The quantitative analyses of phosphorylated AMPK and LC3B-II/LC3B-I were shown in the graphs. **(C)** Huh7 and Hep3B cells were transfected with GFP-LC3B expression vectors and then treated with sorafenib at the indicated concentrations for 6 h. The number of LC3B puncta was evaluated under a fluorescence microscope. The data are shown as the mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by Student's *t*-test.

sorafenib can activate CEBPD expression in Huh7 cells but not in Hep3B cells (**Figures 4A,B**). In addition, the LC3B-II/LC3B-I ratio and the level of caspase-3 activation were significantly lower and the cell viability inhibition effect was minor in Hep3B cells than in Huh7 cells upon sorafenib treatment (**Figures 4B,C**). To verify whether CEBPD is involved in the sorafenib-induced anti-cancer effect, a loss-of-function assay was conducted by reducing the levels of CEBPD with shRNA. The results showed that the loss of CEBPD attenuated the sorafenib-induced increase in LC3B-I/-II conversion and caspase-3 activity (**Figure 4B**) and suppressed the sorafenib-induced inhibition in cell viability (**Figure 4C**) in Huh7 cells. Treatment with an autophagy inhibitor (chloroquine, CQ) also restored sorafenib-inhibited Huh7 cell viability (**Figure 4C**). Our previous studies suggested that the methylation status of the *CEBPD* promoter determines CEBPD induction and expression in HCC and other cancer types (Ko et al., 2008; Li et al., 2015; Chu et al., 2017). However, the methylation states of the *CEBPD* promoter were not different between Huh7 and Hep3B cells (**Supplementary Figure 3**), indicating that a non-DNA methylation mechanism contributes to CEBPD desensitization in liver cancer cells. AMPK is involved in CEBPD activation (Tsai et al., 2017), and the p38 MAPK/cAMP-responsive element binding protein (CREB) pathway is

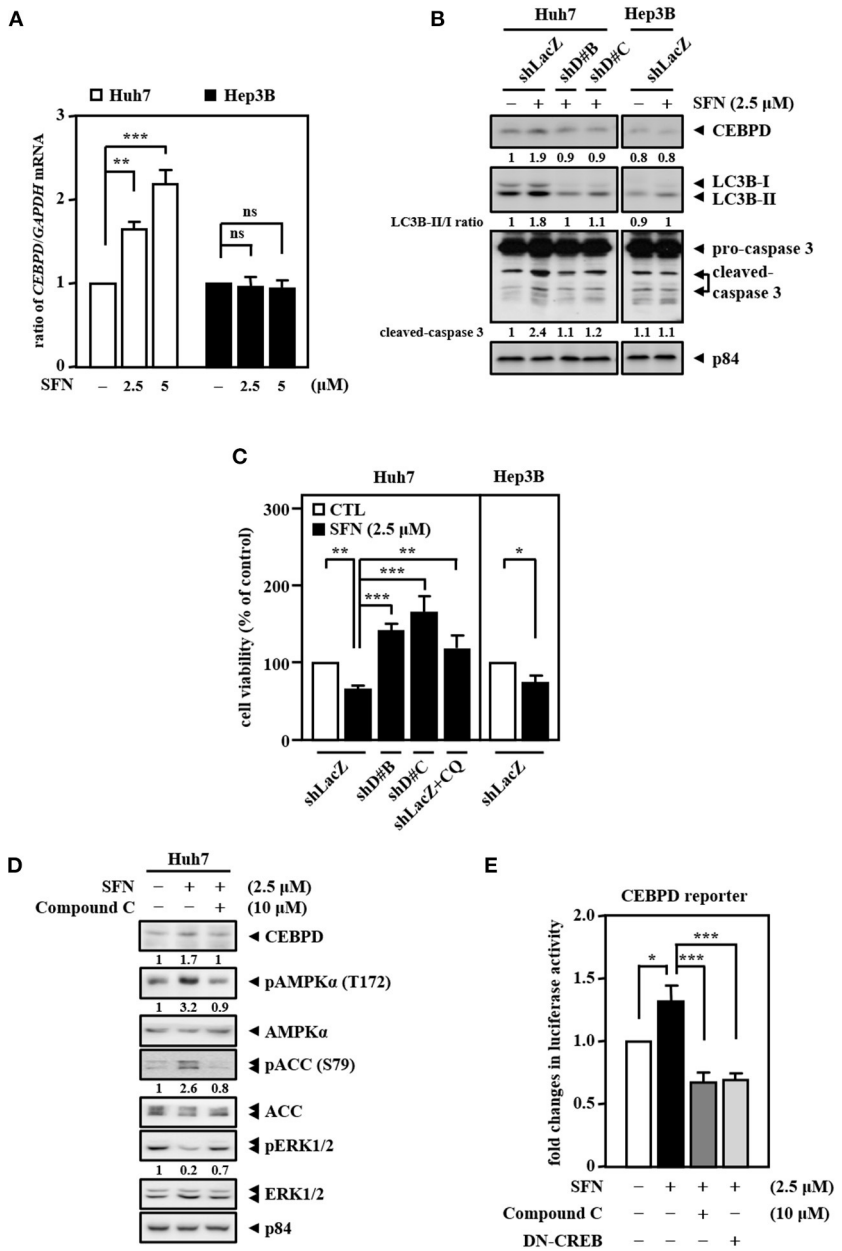


FIGURE 4 | CEBPD is involved in sorafenib-induced autophagic cell death. **(A)** Huh7 and Hep3B cells were treated with sorafenib at the indicated concentrations for 3 and 24 h. Total RNA was harvested for RT-qPCR assays. **(B)** Huh7 and Hep3B cells were infected with lentiviruses encoding shLacZ (shZ) or shCEBPD (shDB and shDC) and then treated with or without sorafenib (2.5 μM). Whole cell lysates were harvested for Western blot analyses. **(C)** Huh7 and Hep3B cells were infected with lentiviruses encoding shLacZ (shZ) or shCEBPD (shDB or shDC) for 3 days. Huh7 cells were pretreated with or without chloroquine (CQ, 10 μM) for 30 min. After being treated with sorafenib (2.5 μM) for 48 h, the cell viability of the infected experimental cells was measured by MTT assays. **(D)** Huh7 cells were pretreated with or without the AMPK inhibitor compound C (10 μM) for 0.5 h and then treated with or without sorafenib (2.5 μM) for an additional 6 h. Whole cell lysates were harvested for Western blot analyses. **(E)** Huh7 cells transfected with *CEBPD* reporters were cotransfected with or without DN-CREB expression vectors for 18 h or treated with or without the AMPK inhibitor compound C (10 μM) for 30 min and then treated with or without sorafenib (2.5 μM) for an additional 3 h. The lysates of the transfected cells were harvested for luciferase assays. The data are shown as the mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by Student's *t*-test.

important for the transcriptional activation of the *CEBPD* gene (Hsiao et al., 2013; Lai et al., 2017). In addition, activation of AMPK has been reported to activate CREB in liver cancer cells (Irungbam et al., 2020). We next tested whether AMPK contributes to sorafenib-induced CEBPD expression. The results demonstrated that the AMPK inhibitor compound C suppressed sorafenib-induced AMPK and ACC phosphorylation as well as CEBPD expression in Huh7 cells (**Figure 4D**). Moreover,

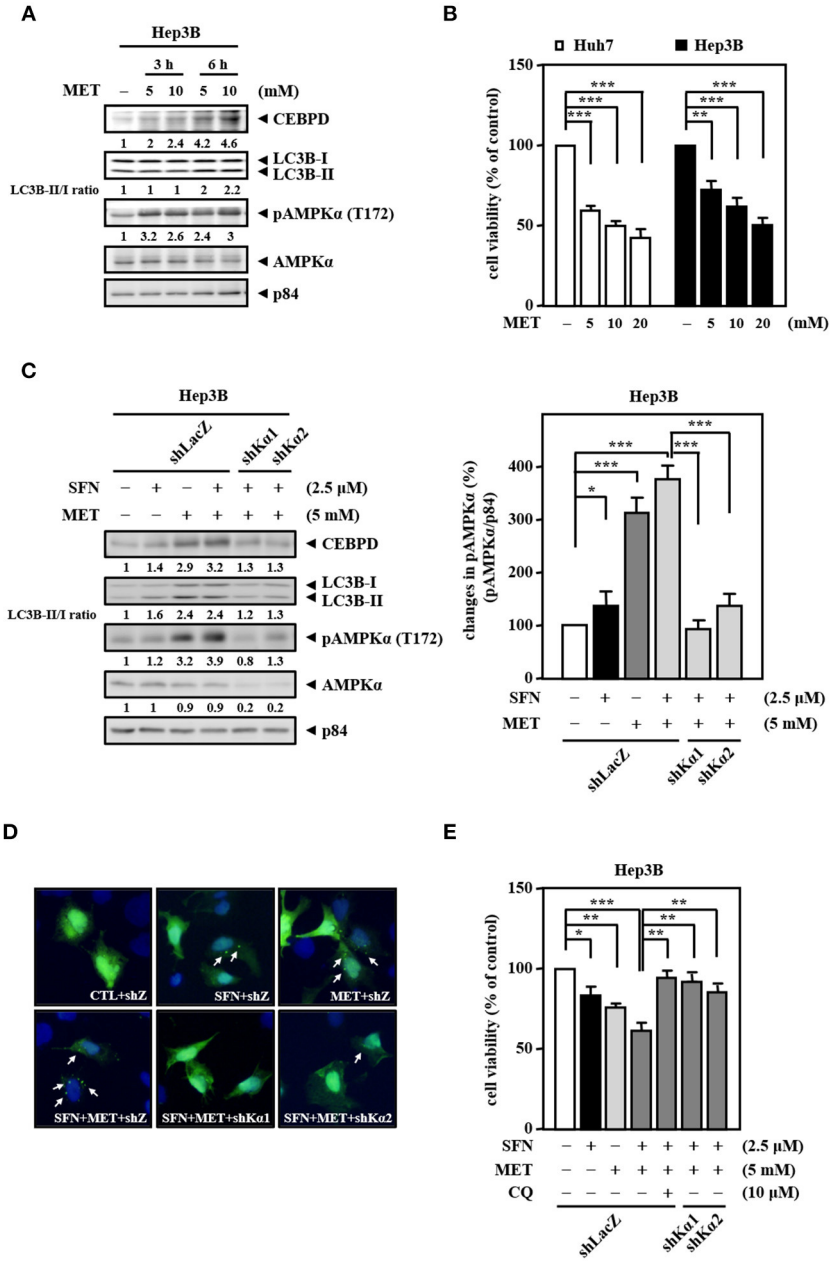


FIGURE 5 | Metformin improves the sensitivity of Hep3B cells to sorafenib. **(A)** Hep3B cells were treated with metformin at the indicated concentrations for 3 and 6 h. Whole cell lysates were harvested for Western blot analyses. **(B)** Huh7 and Hep3B cells were treated with metformin (MET) at the indicated concentrations for 48 h. The cell viability of the experimental cells was measured by MTT assays after 48 h of metformin treatment at the indicated concentrations. **(C)** Hep3B cells were infected with lentiviruses encoding shLacZ (shZ) or shAMPKα (shKα1 or shKα2) for 3 days. Hep3B cells were treated with sorafenib (2.5 μM) or metformin (5 mM) alone or with the combination of sorafenib (2.5 μM) and metformin (5 mM) for 24 h. Whole cell lysates were harvested for Western blot analyses. The quantitative analysis of phosphorylated AMPK was shown in the graph. **(D)** Hep3B cells were transfected with GFP-LC3B expression vectors and then treated with sorafenib or metformin alone or with the combination of sorafenib and metformin for 6 h. The number of LC3B puncta was evaluated under a fluorescence microscope. **(E)** Hep3B cells were pretreated with or without chloroquine (CQ, 10 μM) for 30 min and then treated with sorafenib or metformin alone or with the combination of sorafenib and metformin for 48 h. The cell viability of the experimental cells was measured by MTT assays. The data are shown as the mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by Student's *t*-test.

sorafenib-induced *CEBPD* reporter activity was attenuated in compound C-treated and dominant negative CREB (DN-CREB)-transfected Huh7 cells (**Figure 4E**). Taken together, these results suggest that AMPK participates in sorafenib-induced CEBPD expression, which contributes to autophagic cell death in HCC.

Metformin Improves the Sensitivity of Hep3B Cells to Sorafenib

Our current results suggest that sorafenib cannot efficiently induce AMPK activation to contribute to autophagic cell death due to EGFR overexpression. Since clinical drug metformin can activate AMPK bypassing the inhibitory effect of the EGFR/ERK pathway, we further assess the activity of AMPK and CEBPD in response to metformin for sorafenib resensitization. We first examined the effect of metformin on the activity of AMPK in Hep3B cells. The results revealed that metformin increased AMPK phosphorylation, CEBPD expression, and the LC3B-II/LC3B-I ratio in Hep3B cells (**Figure 5A**). Next, we tested whether metformin inhibits Hep3B and Huh7 cell proliferation. The results revealed that metformin reduced Huh7 and Hep3B cell viability (**Figure 5B**). Moreover, a combination of sorafenib and metformin was used to assess whether metformin can enhance sorafenib sensitivity in Hep3B cells. The results revealed that, compared to sorafenib treatment, combination of sorafenib and metformin significantly enhanced AMPK phosphorylation, CEBPD expression, the LC3B-II/LC3B-I ratio (**Figure 5C**), and the number of LC3B puncta (**Figure 5D**) in Hep3B cells. To further support the contribution of AMPK activity to downstream targets and biological effects, a loss-of-function assay using lentiviruses encoding shAMPKα1 and shAMPKα2 was conducted. The results showed that the knockdown of AMPKα could suppress dual treatment-induced CEBPD expression, LC3B-I/-II conversion (**Figure 5C**), and the number of LC3B puncta (**Figure 5D**) in Hep3B cells. Moreover, the combination treatment reduced Hep3B cell viability more than treatment with sorafenib or metformin alone, and either AMPKα knockdown or autophagy inhibition could also restore dual treatment-inhibited Hep3B cell viability (**Figure 5E**). Collectively, these results suggest that metformin can enhance the death of sorafenib-insensitive EGFR-overexpressed liver cancer cells by activating AMPK/CEBPD-induced autophagy *in vitro*.

The Combination of Sorafenib and Metformin Elicits a Stronger Anti-tumor Effect in a Hep3B Cell Xenograft Mouse Model

We further assessed the *in vivo* effect of the dual treatment of sorafenib and metformin in a human tumor xenograft mouse model in accordance with the ARRIVE guidelines (**Supplementary Table 1**). Concerning the effects of sorafenib dose on toxicity in HCC, we used a relatively lower dose of sorafenib in combination with metformin in Hep3B cell xenografts in NOD/SCID mice. Consistent with the above *in vitro* results, the combined treatment of sorafenib and metformin significantly enhanced cytotoxicity compared with that induced by sorafenib or metformin treatment alone (**Figure 6A**, left panel). Importantly, the combined treatment was well tolerated as evidenced by no weight loss was observed after treatment (**Figure 6A**, right panel). Furthermore, the loss of CEBPD attenuated the combined treatment-induced enhancement of Hep3B tumor xenograft death in NOD-SCID mice (**Figure 6B**, compare lane 1 with lane 3 and lane 3 with lane 4), suggesting that CEBPD has a strong anti-tumor effect. Importantly, the LC3B-II/LC3B-I ratio was examined in tumor lysates extracted from these experimental xenografts. The result demonstrated that the LC3B-II/LC3B-I ratio was induced in metformin treatment alone and in combination group (**Figure 6C**). Collectively, these results suggest that the insufficient activation of autophagy may enable residual HCC cells to survive; however, strong autophagy can contribute to cell death and resensitize sorafenib-resistant HCC cells.

DISCUSSION

In this study, we showed that EGFR activation is a potential determinant of the primary sorafenib resistance of HCC cells with HBV. However, the clinical results revealed that the addition of erlotinib, an oral tyrosine kinase inhibitor of EGFR with moderate anti-tumor activity against HCC, to sorafenib did not affect the overall survival (Finn, 2013). This could be partially explained by the fact that EGFR inhibitors cannot efficiently induce AMPK activation (Peng et al., 2016), and the insufficient activation of autophagy may enable residual cancer cells to resist chemotherapy. Our current results reveal that AMPK and CEBPD are unresponsive to sorafenib due to sustained EGFR/ERK activation in Hep3B cells. Therefore, metformin that has the direct effect on the activity of AMPK and CEBPD may be a potential combined with sorafenib to overcome sorafenib resistance in HCC. Interestingly, accumulation of evidence showed that metformin synergistically sensitizes leukemia cells (Wang et al., 2015) and lung cancer cells (Groenendijk et al., 2015) to sorafenib through AMPK activation, which are consistent with our findings.

There are other downstream signaling pathways regulated by EGFR, including Src/signal transducer and activator of transcription (STAT) and phosphatidylinositol 3-kinase (PI3K)/Akt signal transduction pathways (Nyati et al., 2006). Our previous finding revealed that metformin can reduce Src-mediated CEBPD protein degradation (Tsai et al., 2017). However, Western blot analyses revealed that sorafenib activates Src. Meanwhile, the AMPK inhibitor compound C has no effect on sorafenib-induced Src phosphorylation (**Supplementary Figure 4**). These results suggest that metformin could work via the Src-dependent pathway to enhance CEBPD expression and autophagic cell death in sorafenib-resistant liver cancer cells. Moreover, several studies have demonstrated that metformin inhibits STAT (Feng et al., 2014) and the PI3K/Akt pathway (Pernicova and Korbonits, 2014). Therefore, the application of metformin for the improvement of the efficacy of sorafenib in HCC with EGFR overexpression involves multiple factors that need to be further investigated.

Acute inflammation is a strong and rapid response to tissue injury and protects body, but low-grade and chronic inflammation can be harmful. Sustained cell growth in an inflammatory environment combined with accumulation of genetic abnormalities contributes to cancer progression. Our previous study demonstrated that inflammation-responsive transcription factor CEBPD can induce genomic instability

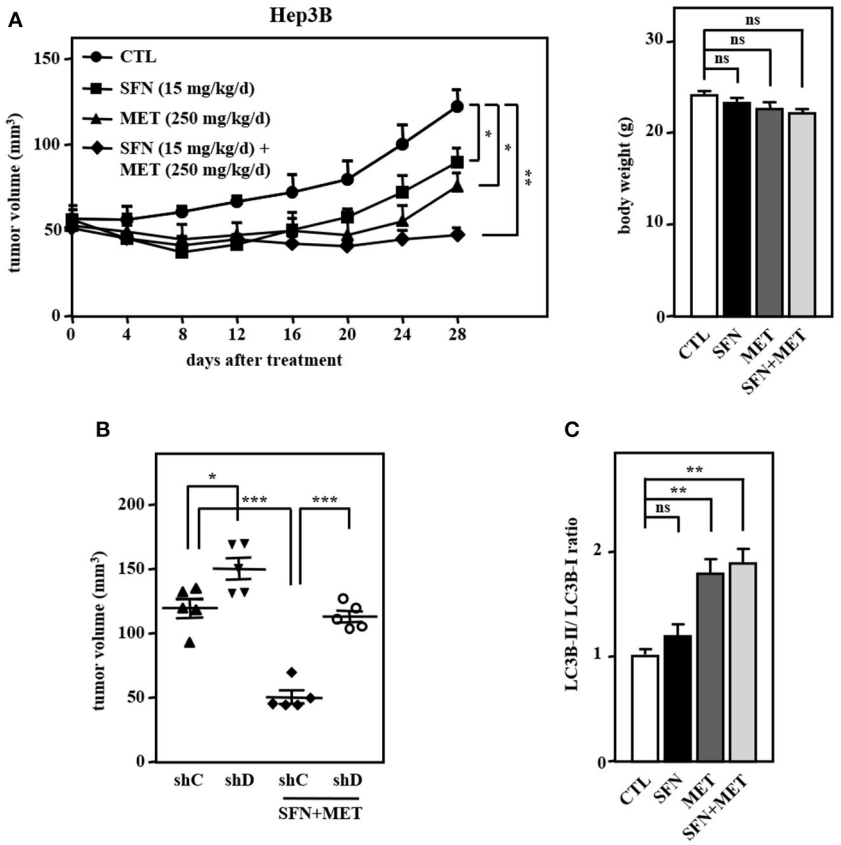


FIGURE 6 | The combination of sorafenib and metformin elicits stronger cytotoxicity in a Hep3B cell xenograft mouse model. Hep3B cells were subcutaneously inoculated into NOD-SCID mice, and the mice then received an intraperitoneal injection of vehicle, sorafenib (15 mg/kg/day), metformin (250 mg/kg/day), or sorafenib (15 mg/kg/day) combined with metformin (250 mg/kg/day). **(A)** Tumor dimensions were obtained at the indicated time points. Following 4 weeks of drug treatment, the mice were sacrificed, and animal weights were obtained. **(B)** Hep3B cells were infected with lentiviruses to drive the stable expression of either IPTG-inducible LacZ shRNA (shC) or IPTG-inducible CEBPD shRNA (shD). Infected Hep3B cells were subcutaneously inoculated into the dorsum of 6-week-old NOD-SCID mice ($n = 5$), and the mice then received an intraperitoneal injection of 200 μl IPTG (0.53 mmol) every other day. The mice were then treated with or without sorafenib (15 mg/kg/day) combined with metformin (250 mg/kg/day) via intraperitoneal injection. After 28 days of treatment, the mice were sacrificed, and the tumor volume was measured. **(C)** After 28-day treatment, the lysates extracted from these experimental xenograft tumors were collected and analyzed by Western blotting. The data are shown as the mean ± SD. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by Student's *t*-test.

and promote tumorigenesis, even though it serves as a tumor suppressor in cervical cancer (Wu et al., 2011). Recent study indicated that the acquired sorafenib resistance may also be associated with genomic instability (Xia et al., 2020). Therefore, the association of insufficient but sustained CEBPD and autophagic activity with genomic instability and cell survival, respectively, in response to sorafenib deserves to be clarified. In this way, the molecular details of how sorafenib establishes acquired resistance will be dissected. The dual roles of CEBPD may orchestrate the dual functions of autophagy to contribute to both death and resistance of cancer cells and this could in part underlie the complex role of inflammation in cancer development.

We propose that metformin, an AMPK activator, restores the sensitivity of EGFR-overexpressed liver cancer cells to sorafenib. However, many anti-diabetic drugs and small molecule compounds should be tested with the goal of activating AMPK in cancer cells. Insulin-sensitizing thiazolidinediones (TZDs) are potent agonist ligands for the nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ). They are also thought to exert some of their anti-diabetic effects through AMPK activation in a variety of tissues, including skeletal muscle (LeBrasseur et al., 2006) and liver (Saha et al., 2004). Glucagon-like peptide-1 (GLP-1) mimetics stimulate insulin secretion in a glucose-dependent manner. Previous studies have shown that these compounds and endogenous GLP-1 can activate the AMPK pathway (Svegliati-Baroni et al., 2011). The first direct AMPK activator was A-769662, but this compound is unsuitable to be used due to its poor oral absorption. Recently, compound 991 is significantly more potent than A-769662 in allosterically activating AMPK (Xiao et al., 2013). Although further clinical trials are needed to evaluate the safety and efficacy of these compounds, our results indicate that therapeutic AMPK

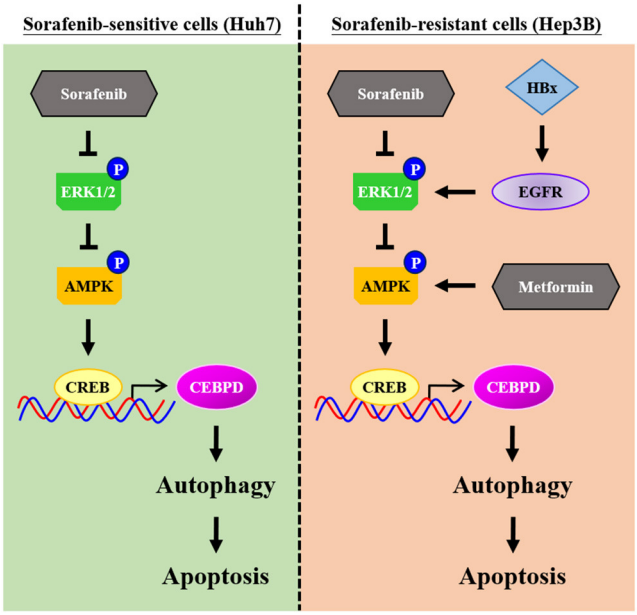


FIGURE 7 | Schematic model of the molecular mechanism by which metformin increases the sensitivity of Hep3B cells to sorafenib. Hep3B cells are intrinsically more resistant than Huh7 cells to sorafenib. High activity of epidermal growth factor receptor (EGFR) and low autophagic responsiveness may determine the primary resistance of Hep3B cells to sorafenib. CCAAT/enhancer binding protein delta (CEBPD), a potent tumor suppressor, is responsive to metformin via AMP-activated protein kinase (AMPK) activation, and it promotes autophagic cell death. Furthermore, metformin can resensitize sorafenib-induced autophagic cell death in Hep3B cells. Taken together, our results provide possible implications for improving the efficacy of sorafenib and helping to develop personalized medicine strategies for HCC patients.

activation should be an attractive target for improving the efficacy of sorafenib.

CONCLUSIONS

Even though new chemotherapy agents are being developed quickly, all chemotherapy agents face the challenge of drug resistance. Primary drug resistance is one of the reasons for the attenuation of the efficacy of chemotherapy agents. In the current study, our results revealed a new insight that insufficient AMPK and CEBPD activation as well as lower autophagic activity play a functional role in sorafenib resistance in liver cancer cells with EGFR overexpression. Meanwhile, we further demonstrated that metformin may be combined with sorafenib to strengthen autophagic cell death (**Figure 7**). The discoveries indicated that AMPK activators and autophagy activators could be potential candidates for further application in sorafenib-resistant liver cancers.

DATA AVAILABILITY STATEMENT

The dataset analysed during the current study is available in the public dataset GSE83148 from GEO (National Center for Biotechnology Information, Bethesda, MD).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Chi-Mei Medical Center in Taiwan (IRB: 10702-E04). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of College of Medicine, National Cheng Kung University (Approval Number: 103209).

AUTHOR CONTRIBUTIONS

H-YLa, H-HT, C-FL, and J-MW: conceptualization. H-YLa, H-HT, C-JY, L-YH, C-CY, C-HH, H-YLi, and F-WC: methodology and formal analysis. H-YLa, H-HT, C-JY, L-YH, C-CY, and C-HH: investigation. H-YLa, H-HT, C-JY, L-YH, C-CY, C-HH, C-FL, and J-MW: resources. H-YLa and H-HT: validation, visualization, and writing–original draft. H-YLa and J-MW: writing–review & editing. J-MW: funding acquisition and supervision. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by grant MOST109-2320-B-006-054- from the Ministry of Science and Technology.

ACKNOWLEDGMENTS

We are grateful to Yi-Chen Chen from the Health Data Science Center at National Cheng Kung University Hospital for providing administrative and technical supports. We thank I-Chen Peng from the Department of Life Sciences at National Cheng Kung University for providing shAMPKα expression vectors.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.596655/full#supplementary-material

Supplementary Figure 1 | EGFR upregulation is associated with lower CEBPD and LC3B levels in *HBV*-infected livers. **(A)** Quantitative data of *EGFR* transcripts were extracted from the transcript microarray of 6 healthy livers and 122 *HBV*-infected livers. **(B)** *HBV*-infected liver tissues were divided into two groups according to the median value of EGFR levels ($EGFR^{low}$ and $EGFR^{high}$). Quantitative data of *CEBPD* and *LC3B* transcripts were extracted from the transcript microarray of $EGFR^{low}$ and $EGFR^{high}$ *HBV*-infected livers.

Supplementary Figure 2 | CEBPD and LC3B are co-downregulated in tumors of HBx transgenic mice. The liver specimens [tumors (T) and adjacent non-tumor (N) tissues] from the 18-month old HBx transgenic mice were analyzed by immunohistochemistry using CEBPD and LC3B antibodies.

Supplementary Figure 3 | Methylation states on the *CEBPD* promoter are not different in Huh7 and Hep3B cells. The CpG methylation status of *CEBPD* promoters in Huh7 and Hep3B cells was determined using MSP assays.

Supplementary Figure 4 | The effects of sorafenib and the AMPK inhibitor compound C on Src phosphorylation in liver cancer cells. **(A)** Two types of liver cancer cells (Huh7 and Hep3B) were treated with or without sorafenib (2.5 μM) for 24 h and harvested for Western blot analyses. **(B)** Huh7 cells were pretreated with or without the AMPK inhibitor compound C (10 μM) for 0.5 h and then treated with or without sorafenib (2.5 μM) for an additional 6 h. Whole cell lysates were harvested for Western blot analyses and examined indicated proteins with specific antibodies.

Supplementary Table 1 | Animal studies comply with the ARRIVE guidelines.

REFERENCES

Agrawal, S., Hofmann, W. K., Tidow, N., Ehrich, M., van den Boom, D., Koschmieder, S., et al. (2007). The C/EBPdelta tumor suppressor is silenced by hypermethylation in acute myeloid leukemia. *Blood* 109, 3895–3905. doi: 10.1182/blood-2006-08-040147

Bray, F., and Parkin, D. M. (2009). Evaluation of data quality in the cancer registry: principles and methods. *Part I: comparability, validity and timeliness. Eur. J. Cancer* 45, 747–755. doi: 10.1016/j.ejca.2008.11.032

Buckley, A. F., Burgart, L. J., Sahai, V., and Kakar, S. (2008). Epidermal growth factor receptor expression and gene copy number in conventional hepatocellular carcinoma. *Am. J. Clin. Pathol.* 129, 245–251. doi: 10.1309/WF10QAAED3PP93BH

Cervello, M., McCubrey, J. A., Cusimano, A., Lampiasi, N., Azzolina, A., and Montalto, G. (2012). Targeted therapy for hepatocellular carcinoma: novel agents on the horizon. *Oncotarget* 3, 236–260. doi: 10.18632/oncotarget.466

Chan, K. M., Wu, T. H., Cheng, C. H., Lee, C. F., Wu, T. J., Chou, H. S., et al. (2019). Implementation of sorafenib treatment for advanced hepatocellular carcinoma: an illustration of current practice in Taiwan. *Cancer Manag. Res.* 11, 1013–1021. doi: 10.2147/CMAR.S186678

Chang, L. H., Huang, H. S., Wu, P. T., Jou, I. M., Pan, M. H., Chang, W. C., et al. (2012). Role of macrophage CCAAT/enhancer binding protein delta in the pathogenesis of rheumatoid arthritis in collagen-induced arthritic mice. *PLoS ONE* 7:e45378. doi: 10.1371/journal.pone.0045378

Chen, H. P., Shieh, J. J., Chang, C. C., Chen, T. T., Lin, J. T., Wu, M. S., et al. (2013). Metformin decreases hepatocellular carcinoma risk in a dose-dependent manner: population-based and *in vitro* studies. *Gut* 62, 606–615. doi: 10.1136/gutjnl-2011-301708

Chiang, C. J., Lo, W. C., Yang, Y. W., You, S. L., Chen, C. J., and Lai, M. S. (2016). Incidence and survival of adult cancer patients in Taiwan, 2002-2012. *J. Formos. Med. Assoc.* 115, 1076–1088. doi: 10.1016/j.jfma.2015.10.011

Chu, Y. Y., Ko, C. Y., Wang, S. M., Lin, P. I., Wang, H. Y., Lin, W. C., et al. (2017). Bortezomib-induced miRNAs direct epigenetic silencing of locus genes and trigger apoptosis in leukemia. *Cell Death Dis.* 8:e3167. doi: 10.1038/cddis.2017.520

Esteve-Puig, R., Canals, F., Colome, N., Merlino, G., and Recio, J. A. (2009). Uncoupling of the LKB1-AMPKalpha energy sensor pathway by growth factors and oncogenic BRAF. *PLoS ONE* 4:e4771. doi: 10.1371/journal.pone.0004771

Feng, Y., Ke, C., Tang, Q., Dong, H., Zheng, X., Lin, W., et al. (2014). Metformin promotes autophagy and apoptosis in esophageal squamous cell carcinoma by downregulating Stat3 signaling. *Cell Death Dis.* 5:e1088. doi: 10.1038/cddis.2014.59

Finn, R. S. (2013). Emerging targeted strategies in advanced hepatocellular carcinoma. *Semin. Liver Dis.* 33(Suppl. 1), S11–S19. doi: 10.1055/s-0033-1333632

Groenendijk, F. H., Mellema, W. W., van der Burg, E., Schut, E., Hauptmann, M., Horlings, H. M., et al. (2015). Sorafenib synergizes with metformin in NSCLC through AMPK pathway activation. *Int. J. Cancer.* 136, 1434–1444. doi: 10.1002/ijc.29113

Hsiao, Y. W., Li, C. F., Chi, J. Y., Tseng, J. T., Chang, Y., Hsu, L. J., et al. (2013). CCAAT/enhancer binding protein delta in macrophages contributes to immunosuppression and inhibits phagocytosis in nasopharyngeal carcinoma. *Sci. Signal.* 6:ra59. doi: 10.1126/scisignal.2003648

Irungbam, K., Roderfeld, M., Glimm, H., Hempel, F., Schneider, F., Hehr, L., et al. (2020). Cholestasis impairs hepatic lipid storage via AMPK and CREB signaling in hepatitis B virus surface protein transgenic mice. *Lab. Invest.* 100, 1411–1424. doi: 10.1038/s41374-020-0457-9

Jackson, R., Psarelli, E. E., Berhane, S., Khan, H., and Johnson, P. (2017). Impact of viral status on survival in patients receiving sorafenib for advanced hepatocellular cancer: a meta-analysis of randomized phase III trials. *J. Clin. Oncol.* 35, 622–628. doi: 10.1200/JCO.2016.69.5197

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., et al. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J.* 19, 5720–5728. doi: 10.1093/emboj/19.21.5720

Khan, M., Imam, H., and Siddiqui, A. (2018). Subversion of cellular autophagy during virus infection: Insights from hepatitis B and hepatitis C viruses. *Liver Res.* 2, 146–156. doi: 10.1016/j.livres.2018.09.002

Kim, I., and He, Y. Y. (2013). Targeting the AMP-activated protein kinase for cancer prevention and therapy. *Front. Oncol.* 3:175. doi: 10.3389/fonc.2013.00175

Ko, C. Y., Hsu, H. C., Shen, M. R., Chang, W. C., and Wang, J. M. (2008). Epigenetic silencing of CCAAT/enhancer-binding protein delta activity by YY1/polycomb group/DNA methyltransferase complex. *J. Biol. Chem.* 283, 30919–30932. doi: 10.1074/jbc.M804029200

Lai, H. Y., Hsu, L. W., Tsai, H. H., Lo, Y. C., Yang, S. H., Liu, P. Y., et al. (2017). CCAAT/enhancer-binding protein delta promotes intracellular lipid accumulation in M1 macrophages of vascular lesions. *Cardiovasc. Res.* 113, 1376–1388. doi: 10.1093/cvr/cvx134

LeBrasseur, N. K., Kelly, M., Tsao, T. S., Farmer, S. R., Saha, A. K., Ruderman, N. B., et al. (2006). Thiazolidinediones can rapidly activate AMP-activated protein kinase in mammalian tissues. *Am. J. Physiol. Endocrinol. Metab.* 291, E175–E181. doi: 10.1152/ajpendo.00453.2005

Li, C. F., Tsai, H. H., Ko, C. Y., Pan, Y. C., Yen, C. J., Lai, H. Y., et al. (2015). HMDB and 5-AzadC combination reverses tumor suppressor CCAAT/enhancer-binding protein delta to strengthen the death of liver cancer cells. *Mol. Cancer Ther.* 14, 2623–2633. doi: 10.1158/1535-7163.MCT-15-0025

Liang, C., Feng, P., Ku, B., Dotan, I., Canaani, D., Oh, B. H., et al. (2006). Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. *Nat. Cell Biol.* 8, 688–699. doi: 10.1038/ncb1426

Liu, Y., and Levine, B. (2015). Autosis and autophagic cell death: the dark side of autophagy. *Cell Death Differ.* 22, 367–376. doi: 10.1038/cdd.2014.143

Lu, L. C., Chen, P. J., Yeh, Y. C., Hsu, C. H., Chen, H. M., Lai, M. S., et al. (2017). Prescription Patterns of sorafenib and outcomes of patients with advanced hepatocellular carcinoma: a national population study. *Anticancer Res.* 37, 2593–2599. doi: 10.21873/anticanres.11604

Nyati, M. K., Morgan, M. A., Feng, F. Y., and Lawrence, T. S. (2006). Integration of EGFR inhibitors with radiochemotherapy. *Nat. Rev. Cancer* 6, 876–885. doi: 10.1038/nrc1953

Ochi, M., Otsuka, M., Maruyama, R., and Koike, K. (2020). HBx increases EGFR expression by inhibiting miR129-5p function. *Biochem. Biophys. Res. Commun.* 529, 198–203. doi: 10.1016/j.bbrc.2020.06.018

O'Rourke, J., Yuan, R., and DeWille, J. (1997). CCAAT/enhancer-binding protein-delta (C/EBP-delta) is induced in growth-arrested mouse mammary epithelial cells. *J. Biol. Chem.* 272, 6291–6296. doi: 10.1074/jbc.272.10.6291

Pan, Y. C., Li, C. F., Ko, C. Y., Pan, M. H., Chen, P. J., Tseng, J. T., et al. (2010). CEBPD reverses RB/E2F1-mediated gene repression and participates in HMDB-induced apoptosis of cancer cells. *Clin. Cancer Res.* 16, 5770–5780. doi: 10.1158/1078-0432.CCR-10-1025

Park, M. A., Reinehr, R., Haussinger, D., Voelkel-Johnson, C., Ogretmen, B., Yacoub, A., et al. (2010). Sorafenib activates CD95 and promotes autophagy and cell death via Src family kinases in gastrointestinal tumor cells. *Mol. Cancer Ther.* 9, 2220–2231. doi: 10.1158/1535-7163.MCT-10-0274

Peng, M., Huang, Y., Tao, T., Peng, C. Y., Su, Q., Xu, W., et al. (2016). Metformin and gefitinib cooperate to inhibit bladder cancer growth via

both AMPK and EGFR pathways joining at Akt and Erk. *Sci. Rep*. 6:28611. doi: 10.1038/srep28611

Pernicova, I., and Korbonits, M. (2014). Metformin–mode of action and clinical implications for diabetes and cancer. *Nat. Rev. Endocrinol*. 10, 143–156. doi: 10.1038/nrendo.2013.256

Saha, A. K., Avilucea, P. R., Ye, J. M., Assifi, M. M., Kraegen, E. W., and Ruderman, N. B. (2004). Pioglitazone treatment activates AMP-activated protein kinase in rat liver and adipose tissue *in vivo*. *Biochem. Biophys. Res. Commun*. 314, 580–585. doi: 10.1016/j.bbrc.2003.12.120

Shi, W. Y., Xiao, D., Wang, L., Dong, L. H., Yan, Z. X., Shen, Z. X., et al. (2012). Therapeutic metformin/AMPK activation blocked lymphoma cell growth via inhibition of mTOR pathway and induction of autophagy. *Cell Death Dis*. 3:e275. doi: 10.1038/cddis.2012.13

Sivko, G. S., and DeWille, J. W. (2004). CCAAT/Enhancer binding protein delta (c/EBPdelta) regulation and expression in human mammary epithelial cells: I. *"Loss of function" alterations in the c/EBPdelta growth inhibitory pathway in breast cancer cell lines. J. Cell. Biochem*. 93, 830–843. doi: 10.1002/jcb.20223

Svegliati-Baroni, G., Saccomanno, S., Rychlicki, C., Agostinelli, L., De Minicis, S., Candelaresi, C., et al. (2011). Glucagon-like peptide-1 receptor activation stimulates hepatic lipid oxidation and restores hepatic signalling alteration induced by a high-fat diet in nonalcoholic steatohepatitis. *Liver Int*. 31, 1285–1297. doi: 10.1111/j.1478-3231.2011.02462.x

Takamura, A., Komatsu, M., Hara, T., Sakamoto, A., Kishi, C., Waguri, S., et al. (2011). Autophagy-deficient mice develop multiple liver tumors. *Genes Dev*. 25, 795–800. doi: 10.1101/gad.2016211

Tomic, T., Botton, T., Cerezo, M., Robert, G., Luciano, F., Puissant, A., et al. (2011). Metformin inhibits melanoma development through autophagy and apoptosis mechanisms. *Cell Death Dis*. 2:e199. doi: 10.1038/cddis.2011.86

Tsai, H. H., Lai, H. Y., Chen, Y. C., Li, C. F., Huang, H. S., Liu, H. S., et al. (2017). Metformin promotes apoptosis in hepatocellular carcinoma through the CEBPD-induced autophagy pathway. *Oncotarget* 8, 13832–13845. doi: 10.18632/oncotarget.14640

Wang, F., Liu, Z., Zeng, J., Zhu, H., Li, J., Cheng, X., et al. (2015). Metformin synergistically sensitizes FLT3-ITD-positive acute myeloid leukemia to sorafenib by promoting mTOR-mediated apoptosis and autophagy. *Leuk. Res*. 39, 1421–1427. doi: 10.1016/j.leukres.2015.09.016

Wang, J. M., Tseng, J. T., and Chang, W. C. (2005). Induction of human NF-IL6beta by epidermal growth factor is mediated through the p38 signaling pathway and cAMP response element-binding protein activation in A431 cells. *Mol. Biol. Cell*. 16, 3365–3376. doi: 10.1091/mbc.e05-02-0105

Wang, W. J., Li, C. F., Chu, Y. Y., Wang, Y. H., Hour, T. C., Yen, C. J., et al. (2017). Inhibition of the EGFR/STAT3/CEBPD axis reverses cisplatin cross-resistance with paclitaxel in the urothelial carcinoma of the urinary bladder. *Clin. Cancer Res*. 23, 503–513. doi: 10.1158/1078-0432.CCR-15-1169

Wang, X., Li, W., Zhang, N., Zheng, X., and Jing, Z. (2019). Opportunities and challenges of co-targeting epidermal growth factor receptor and autophagy signaling in non-small cell lung cancer. *Oncol. Lett*. 18, 499–506. doi: 10.3892/ol.2019.10372

Wang, Z., Han, W., Sui, X., Fang, Y., and Pan, H. (2014). Autophagy: a novel therapeutic target for hepatocarcinoma (review). *Oncol. Lett*. 7, 1345–1351. doi: 10.3892/ol.2014.1916

Wu, D. H., Jia, C. C., Chen, J., Lin, Z. X., Ruan, D. Y., Li, X., et al. (2014). Autophagic LC3B overexpression correlates with malignant progression and predicts a poor prognosis in hepatocellular carcinoma. *Tumour Biol*. 35, 12225–12233. doi: 10.1007/s13277-014-2531-7

Wu, S. R., Li, C. F., Hung, L. Y., Huang, A. M., Tseng, J. T., Tsou, J. H., et al. (2011). CCAAT/enhancer-binding protein delta mediates tumor necrosis factor alpha-induced Aurora kinase C transcription and promotes genomic instability. *J. Biol. Chem*. 286, 28662–28670. doi: 10.1074/jbc.M111.270710

Xia, S., Pan, Y., Liang, Y., Xu, J., and Cai, X. (2020). The microenvironmental and metabolic aspects of sorafenib resistance in hepatocellular carcinoma. *EBioMedicine* 51:102610. doi: 10.1016/j.ebiom.2019.102610

Xiao, B., Sanders, M. J., Carmena, D., Bright, N. J., Haire, L. F., Underwood, E., et al. (2013). Structural basis of AMPK regulation by small molecule activators. *Nat. Commun*. 4:3017. doi: 10.1038/ncomms4017

Zhu, Y. J., Zheng, B., Wang, H. Y., and Chen, L. (2017). New knowledge of the mechanisms of sorafenib resistance in liver cancer. *Acta Pharmacol. Sin*. 38, 614–622. doi: 10.1038/aps.2017.5

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Lai, Tsai, Yen, Hung, Yang, Ho, Liang, Chen, Li and Wang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Oncology

REVIEW
published: 01 February 2021
doi: 10.3389/fonc.2020.619727

Pro-Tumoral Functions of Autophagy Receptors in the Modulation of Cancer Progression

Cristóbal Cerda-Troncoso[1], *Manuel Varas-Godoy*[1*] *and Patricia V. Burgos*[1,2*]

[1] Centro de Biología Celular y Biomedicina (CEBICEM), Facultad de Medicina y Ciencia, Universidad San Sebastián, Santiago, Chile, [2] Centro de Envejecimiento y Regeneración (CARE-UC), Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

OPEN ACCESS

Edited by:
Waleska Kerllen Martins, Universidade Anhanguera de São Paulo, Brazil

Reviewed by:
Guang-Chao Chen, Academia Sinica, Taiwan
João Agostinho Machado-Neto, University of São Paulo, Brazil

***Correspondence:**
Patricia V. Burgos
patricia.burgos@uss.cl
Manuel Varas-Godoy
manuel.varas@uss.cl

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *21 October 2020*
Accepted: *10 December 2020*
Published: *01 February 2021*

Citation:
Cerda-Troncoso C, Varas-Godoy M and Burgos PV (2021) Pro-Tumoral Functions of Autophagy Receptors in the Modulation of Cancer Progression. Front. Oncol. 10:619727. doi: 10.3389/fonc.2020.619727

Cancer progression involves a variety of pro-tumorigenic biological processes including cell proliferation, migration, invasion, and survival. A cellular pathway implicated in these pro-tumorigenic processes is autophagy, a catabolic route used for recycling of cytoplasmic components to generate macromolecular building blocks and energy, under stress conditions, to remove damaged cellular constituents to adapt to changing nutrient conditions and to maintain cellular homeostasis. During autophagy, cells form a double-membrane sequestering a compartment termed the phagophore, which matures into an autophagosome. Following fusion with the lysosome, the cargo is degraded inside the autolysosomes and the resulting macromolecules released back into the cytosol for reuse. Cancer cells use this recycling system during cancer progression, however the key autophagy players involved in this disease is unclear. Accumulative evidences show that autophagy receptors, crucial players for selective autophagy, are overexpressed during cancer progression, yet the mechanisms whereby pro-tumorigenic biological processes are modulated by these receptors remains unknown. In this review, we summarized the most important findings related with the pro-tumorigenic role of autophagy receptors p62/SQSTM1, NBR1, NDP52, and OPTN in cancer progression. In addition, we showed the most relevant cargos degraded by these receptors that have been shown to function as critical regulators of pro-tumorigenic processes. Finally, we discussed the role of autophagy receptors in the context of the cellular pathways implicated in this disease, such as growth factors signaling, oxidative stress response and apoptosis. In summary, we highlight that autophagy receptors should be considered important players of cancer progression, which could offer a niche for the development of novel diagnosis and cancer treatment strategies.

Keywords: autophagy, autophagy receptors, cancer progression, metastasis, aggressiveness

INTRODUCTION TO PHASES OF CANCER DEVELOPMENT

According with the World Health Organization (WHO) in 2018 around 18.1 million people in the word had cancer, and 9.6 million died due to this disease, making it the second leading cause of death worldwide (1).

The development of cancer, termed carcinogenesis is a multistep process involving three different stages: initiation, promotion, and progression (2, 3) (**Figure 1A**). The tumor

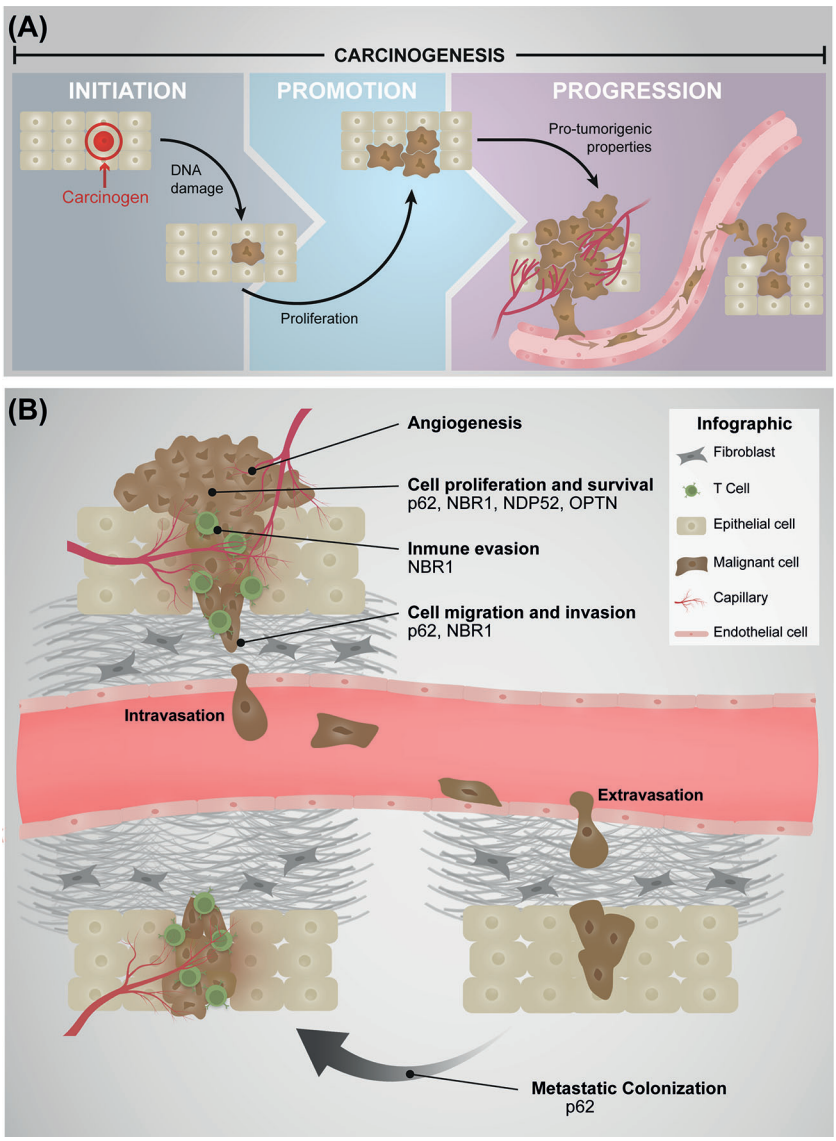


FIGURE 1 | **(A)** Stages of Carcinogenesis. Initiation involves irreversibly alterations of particular tissue cells and increased susceptibility to tumor progression. The alterations are frequently related with mutational events induced with chemicals, radiation or biological agents (carcinogen). Promotion implicates the clonal expansion of altered cells leading to a visible tumor, a stage known to be reversible. In the progression stage, cells show several characteristic processes necessary to develop a malignant phenotype characterized by aggressive properties such as angiogenesis, cell proliferation and survival, immune evasion, cell migration and invasion, and metastasis. **(B)** Involvement of autophagy receptors in cancer progression. During the stage of cancer progression several characteristic processes occur. Angiogenesis corresponds to the formation of interconnected capillaries within the tumor. This is the product of the up-regulation and secretion of pro-angiogenic factors by cancer cells, a crucial process in the supply of oxygen and nutrients to the tumor. Cytosolic autophagy receptors do not promote this process. Cell proliferation and survival are the consequence of genetic changes which promotes metabolic and morphological features that sustain these events. P62, NBR1, NDP52, and OPTN are involved in the promotion of these processes by several mechanisms. Immune evasion corresponds to the mechanism by which cancer cells evaded the immune system, here represented by T cells. NBR1 is known to contribute to immune evasion. Migration and invasion processes are part of the metastatic cascade, in which cells acquire the capacity to migrate and invade the surrounding tissue of the primary tumor. Furthermore, it is proceeded by the intravasation into the circulatory or lymphatic system. p62 and NBR1 receptors promote migration and invasion processes. After intravasation, survival cells in circulation proceed to extravasation in a distant site (respect to primary tumor), and colonize and grow in a new site (metastasis colonization). p62 supports the metastatic process.

initiation and promotion involve irreversible genetic alterations in normal cells, induced by a carcinogen, followed by a reversible process regulated by epigenetic modifications, which promotes the clonal expansion of the altered cells (2) (**Figure 1A**). The final result of these two steps is the generation of a pre-neoplastic lesion forming a visible tumor (2, 4, 5). Although both, initiation and promotion are two crucial steps in cancer development, it is not until the tumor progression step is triggered that altered cells begins to express a malignant phenotype and acquire more aggressive characteristics forming cancer cells (2, 5, 6) (**Figure 1A**). In this early stage of the tumor progression, cancer cells show an increase in the frequency of additional genetic abnormalities such as number of chromosomes, single point mutations, translocations, deletions, and amplifications of genes namely *TP53*, *RB1*, *EGFR*, and *KRAS*, among others (7), which are responsible for promoting metabolic and morphological changes that sustain the proliferation of cancer cells (2, 4, 5, 8) (**Figure 1B**). In addition, to sustain the ability to proliferate, cancer cells must acquire several properties to contribute to the tumor progression, including resistance to cell death, induction of angiogenesis, evasion of the immune system, and activation of the metastatic cascade (9) (**Figure 1B**). During tumor progression, cancer cells are exposed to extreme conditions characteristic of the tumor microenvironment, however how tumor cells adapt to these adverse scenarios is only partially understood. For example, it has been shown that hypoxia, a physiological feature present in the tumor microenvironment triggers apoptosis, dependent of the tumor suppressor p53 (10). Strikingly, the *TP53* gene is frequently found mutated in cancer cells, which elicits a loss of function that ultimately results in apoptosis resistance (11). Cancer cells must also survive to the attack of immune cells, a process known as immune evasion (9) (**Figure 1B**). Here, cancer cells can evade the immune system losing the expression of MHC-I, activating either the intrinsic signaling pathway WNT/β-Catenin axis or promoting the secretion of the factor like VEGF-A, among others (12). In fact, hypoxia up-regulates the expression and secretion of VEGF-A triggering the formation of interconnected capillaries within the tumor (13). This process is called angiogenesis, which is responsible of oxygen and nutrients supply to the growing tumor, allowing cancer cells to survive and proliferate (**Figure 1B**). Angiogenesis is also required to provide an escape route to cancer cells for dissemination and colonization in distant organs through the process of metastases (14). The metastatic cascade involves the capacity of cancer cells present in the primary tumor to migrate and invade the surrounding tissues leading to intravasation in the circulatory or lymphatic system (**Figure 1B**). Cancer cells survive in the circulation, including extravasation in a distant site, with the capacity to colonize and grow in the new site (15–17).

Several cellular and signaling pathways are involved in how pro-tumorigenic properties in cancer cells are triggered, impacting multiple steps in the cascade of tumor progression (9, 18). One cellular pathway originally implicated as a tumor-suppression mechanism is autophagy, which is now considered a potent tumor promoter cellular pathway (19).

AUTOPHAGY: A CRUCIAL CELLULAR PATHWAY DURING CANCER PROGRESSION

Although several studies have shown that basal levels of autophagy can suppress initiation of tumor development (20), a growing number of studies indicated that autophagy enables tumor cell survival, growth, and malignancy by facilitating the supply of metabolic demands during tumor progression (21, 22). In fact, defects in the autophagic machinery often restrain the proliferation, dissemination, and metastatic potential of malignant cells. Indeed, pharmacological interruption of autophagy or genetic knockdown of crucial ATG proteins promoted apoptosis of tumor cells (23–27). In addition, autophagy-deficient tumors are often more sensitive to several chemotherapeutic agents as well as to radiation therapy than their autophagy-proficient counterparts (20, 28, 29). In this review, we summarize the contribution of autophagy cytosolic receptors during the tumor progression stage in carcinogenesis.

Macroautophagy (herein referred to as autophagy) is a catabolic process involving the engulfment of cytoplasmic material into double-membraned autophagosomes that subsequently fused with lysosomes to form autolysosomes, where the materials are finally degraded by lysosomal hydrolytic enzymes (30, 31) (**Figure 2**). Autophagy substrates included abnormal constituents such as protein aggregates, damaged organelles and intracellular pathogens (32). Autophagy is also involved in the degradation of normal cellular constituents for cell survival under restriction of nutrients or by the actions of stressors, a response necessary to maintain cellular fitness in response to environmental conditions contributing to the pathogenesis of various disorders, including cancer (30, 33).

The mechanism of autophagy consists of multiple steps, including formation and expansion of the pre-autophagosomal isolation membrane (phagophore) induced by cellular signals, substrate engulfment, autophagosome closure, and autophagosome-lysosome fusion (30, 34) (**Figure 2**).

Cellular signals promote the formation of the phagophore at specific subdomains of the endoplasmic reticulum (ER) enriched of phosphatidylinositol synthase (35). Within these domains occurs the recruitment of several ATG proteins necessary for early events of phagophore formation and expansion that mediates the synthesis of phosphatidyl-inositol-3-phosphate (PI3P), a pivotal phospholipid needed in the later recruitment of other ATG proteins (34) (**Figure 3**). Key ATGs are members of the yeast Atg8 family of ubiquitin (Ub)-like proteins (LC3A, LC3B, LC3C, GABARAP, GABARPL1, and GABARAPL2 in mammals) which play roles in autophagosome formation and autophagosome-lysosome fusion (36, 37). The best studied member of this family is LC3B (product of the MAP1LC3B), which undergoes conversion from a soluble, cytosolic form (LC3B-I) to a phophatidylethanolamine (PE)-conjugated, membrane-bound form (LC3B-II) (38). LC3B-II subsequently interacts with LC3-interacting region (LIR) motifs of various

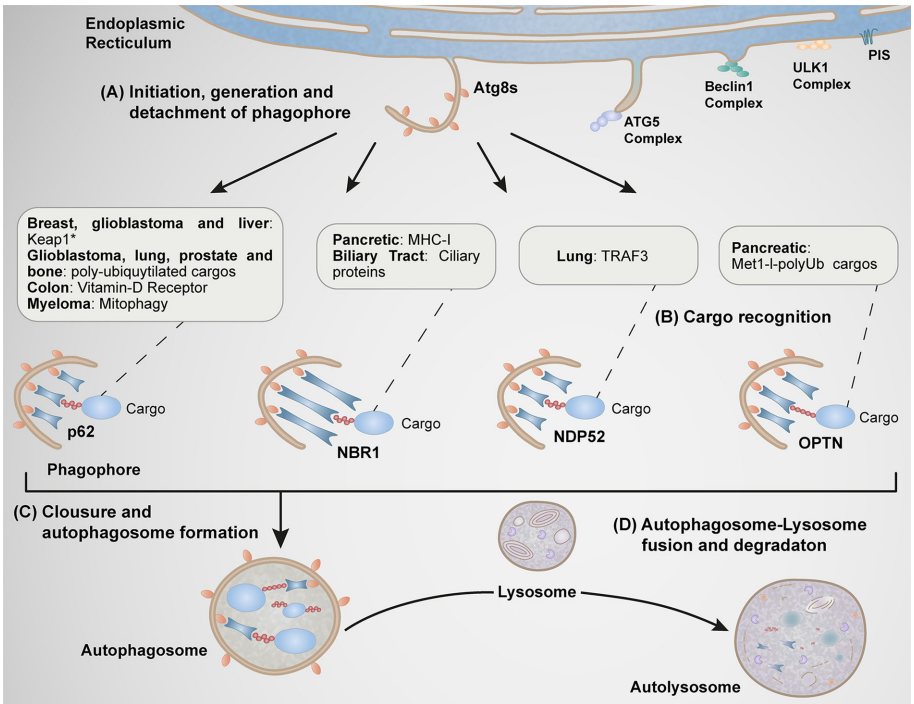


FIGURE 2 | Function of autophagy receptors in different types of cancer. **(A)** At specific subdomains of the endoplasmic reticulum (ER) enriched of phosphatidylinositol synthase (PIS), various Atgs complexes are recruited (ULK1 and Beclin1 complexes). These steps are implicated during early stages of autophagosome formation. Subsequently, the Atg5 complex is recruited to this location facilitating the conversion [from a soluble cytosolic form to a phophatidylethanolamine (PE)-conjugated membrane-bound form] of Atg8 family members (LC3A, LC3B, LC3C, GABARAP, GABARPL1, and GABARAPL2), process implicated in the elongation of the membrane, structure known as phagophore. **(B)** The phagophore is further detached at the ER, where ATG8s proteins begin to interact with autophagy receptors responsible of the selective capture of cargos. **(C)** The final closure of the phagophore form a vesicular double membrane structure called autophagosome. **(D)** The autophagosome finally fuses with the lysosome forming the autolysosome. p62, NBR1, NDP52, and OPTN function in the progression stage of carcinogenesis in several types of cancer by the selective capture of specific cargos indicated in the boxes.

cargo receptors to capture autophagic cargos into forming autophagosomes (39, 40) (**Figures 2**, **3**).

Selective autophagy is orchestrated by cargo receptors responsible for the recognition and incorporation of cargos into the autophagosomes (41). Among these receptors are cytosolic proteins such as p62/SQSTM1, NBR1, OPTN, NDP52, TAX1BP1, and TOLLIP. These receptors bind polyubiquitinated cargos *via* their Ub-binding domains (42, 43). Other cargo receptors are anchored to the autophagic cargos *via* their transmembrane domains, as is the case for BNIP3, NIX and FUNDC1 in mitochondrial autophagy (mitophagy) (39, 40), and RTN3, SEC62, CCPG1, FAM134B and TEX264 in ER autophagy (ER-phagy) (40, 44–48). After fusion of autophagosomes with lysosomes, the autophagy cargos, together with the Atg8-family proteins and cargo receptors, are degraded in autolysosomes (49, 50).

Interestingly, several cytosolic autophagy receptors such as p62/SQSTM1, NBR1, NDP52 and OPTN have been reported to be overexpressed in several types of cancer playing regulatory roles in the last stage of carcinogenesis (**Figures 1**, **2**). In this review, we focus on the emerging roles of autophagy receptors in cancer cell biology.

MOLECULAR FEATURES OF AUTOPHAGY CYTOSOLIC RECEPTORS

Most of cytosolic autophagy receptors are characterized by the presence of specific domains that define their role as cytosolic sensors of damaged cellular constituents. Generally, they harbor both LC3-interactin region (LIR) and ubiquitin-binding domains (UBDs) (51). The LIR motif is considered a hallmark of these receptors corresponding to a short sequence of 6 amino acids based on the multiple alignments of LIR sequences. This sequence is known to be responsible for the interaction with ubiquitin-like proteins like the lipidated ATG8-proteins (LC3s and GABARAPs) in the phagophore membrane (39, 40, 52). UBDs are modular elements found in each autophagy receptor that bind non-covalently to the protein modifier ubiquitin (39, 51). The preferences of UBDs for ubiquitin chains of specific length and linkage are central to their functions in the recognition of cargos into the autophagosomes. Most UBDs use α-helical structures to bind a hydrophobic patch in the β-sheet of ubiquitin (53). For instance, the ubiquitin-binding zinc finger (UBZ) binds ubiquitin with a single α-helix oriented either parallel or antiparallel to the central β-strand. However, other

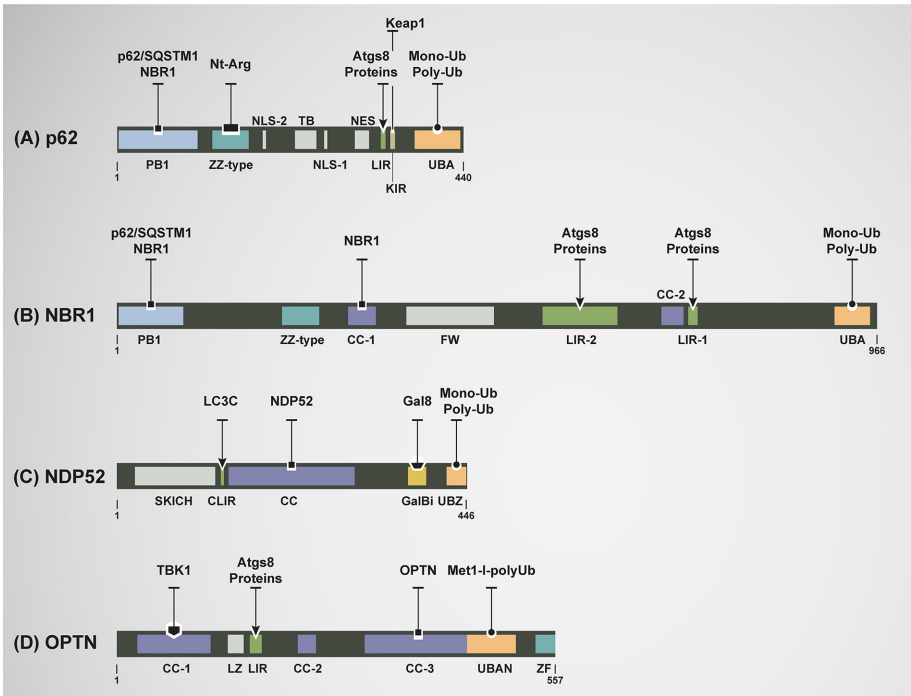


FIGURE 3 | Domain architecture of mammalian autophagy receptors and relevant interactions. **(A)** p62: N-terminal region Phox-BEM1 domain (PB1) mediates p62 homodimerization or its heterodimerization with NBR1; ZZ-type zinc finger domain recognizes N-terminal argenylated substrates (Nt-Arg); nuclear localization signals (NLS1 and NLS2); tumor necrosis factor (TNF) associated receptor-6 (TRAF6) binding (TB) domain; export motif (NES); LC3-interacting region (LIR) motifs mediate the interaction with all Atg8s; KEAP1-interacting region (KIR) binding with KEAP1; and ubiquitin-associated (UBA) domain recognizes mono and poly-ubiquitylated (Mono-Ub and Poly-Ub) substrates. **(B)** NBR1: PB1 mediates interaction with p62 and itself; ZZ-type Zink finger, Coiled-coil-1 (CC-1) mediates self-oligomerization, four tryptophan (FW); LIR-2 motif; CC-2 domain; LIR-1 motif, binding to Atgs8 proteins more functional that LIR-2; and UBA domain recognizes mono-Ub and poly-Ub substrates. **(C)** NDP52: skeletal muscle and kidney-enriched inositol phosphatase carboxyl homology domain (SKICH); LC3C-specific LC3-interacting region (CLIR) mediates selective and strong binding to LC3C; Coiled-coil (CC) domain participates in its homodimerization; Galectin-8 binding region (GalBi) mediates the interaction to Galectin-8 in the context xenophay and lysophagy; and ubiquitin-binding zinc finger (UBZ) domain binds to mono-Ub or poly-Ub. **(D)** OPTN: three Coiled-coil domains are found (CC-1, CC-2, and CC-3). CC-1 domain promotes the formation of a hetero-tetramer complex between OPTN and serine/threonine TANK-binding kinase 1 (TBK1). CC-3 domain mediates the homodimerization of OPTN; a leucine zipper (LZ) domain; LIR motif binds to all members of Atg8s family; ubiquitin-binding domain of ABIN proteins and NEMO (UBAN) binds to methionine1 (Met1)-linked linear polyubiquitin (Met1-l-polyUb) of ubiquitylated cargos; and zinc finger (ZF) domain.

ubiquitin-binding elements, including the ubiquitin-associated (UBA) domain and ubiquitin binding in ABIN and NEMO (UBAN) domain bind ubiquitin through two discontinuous α-helices (53). Interestingly, a variety of post-translational modifications including acetylation, phosphorylation or ubiquitylation can positively regulated the LIRs and UBDs domains enhancing their affinity to ATG8s proteins and ubiquitin, respectively (54–57).

p62/SQSTM1/Sequestosome-1 (Hereafter Referred to as p62)

p62 was the first autophagy receptor identified in mammals (50). This autophagy receptor is a multidomain protein, which contains a LIR motif that interacts with LC3s/GABARAPs attached to the autophagosomes and an UBA domain located in its C-terminal region allowing p62 to associate with ubiquitin and ubiquitin-tagged cargos. This binding results in the formation of cytosolic aggregates and/or the incorporation of cargos into autophagosomes having a functional role in cell survival (58, 59). In addition, p62 contains other additional modules with a role in autophagy such as the ZZ type zinc finger domain. This domain binds to cytosolic cargos bearing amino terminal arginine residues (Nt-Arg) generated by proteolytic processing (N-degrons), interaction that drives these cargos for autophagy degradation (60–63); and a KEAP1-interacting region (KIR) implicated in the sequestration of KEAP1, a key adaptor protein for Cullin-3 ubiquitin ligase implicated in the ubiquitylation and inactivation of the transcription factor NRF2 by degradation through the ubiquitin proteasome system (UPS) (59, 64) (**Figure 3**). Thus, p62 competitively binds to KEAP1 to allow NRF2 function, a transcription factor engaged in the control of ROS levels (65). p62 is expressed in all tissues and has been extensively studied as a scaffold protein in several signal transduction pathways, many of which have been involved in cell survival and cell death (65–68).

p62 and Poor Prognosis in Cancer Patients

p62 has been found overexpressed in different types of tumors, which expression has been associated with poor prognosis in cancer. For example, studies performed in patients with non-small cell lung cancer, including those with lung adenocarcinoma, showed an increase in the levels of p62, correlating with poor prognosis in this type of cancer (69). In addition, immunohistochemistry analysis of tumors derived from patients with non-small cell lung cancer demonstrated an association between high expression of p62 and the aggressiveness of the tumor (70). A similar correlation was also reported in patients with colorectal cancer, osteosarcoma, prostate cancer, hepatocellular carcinoma, breast cancer, and acute myeloid leukemia, among others (71–75).

p62 and Pro-Tumorigenic Properties

The association of p62 expression with the aggressiveness of several types of cancer has been investigated in different cellular models, in which the contribution of p62 in the induction of different pro-tumorigenic properties has been proven. In a cell line of lung adenocarcinoma, silencing of p62 promotes the formation of aberrant autophagosomes, condition that triggers cancer cell death (76). In the same context, reduction in the levels of p62 in a model of chemoresistance of small-cell lung cancer increases its sensitivity against cisplatin. Contrary, overexpression of p62 enhances the resistance to this chemotherapeutic agent, preventing cell death in response to this treatment (77). In the colorectal cancer cell line SW480, p62 proteins levels are found elevated (78), which correlates with active autophagy pathway compared to other cellular models of this type of cancer (79). Interestingly, silencing of p62 in SW480 cell line decreases cell proliferation and their capacity to invade and migrate. Additionally, injection of p62 depleted SW480 cells in mice decreases tumor growth and metastasis into the lung, compared to control cells (78). Similar findings have been reported with F5M2 and F4 cell lines of osteosarcoma, which present high levels of p62 (73). Silencing of p62 in these cell types decreases their proliferative capacity, migration, and invasion (73). Another example is the cell line Huh-1, a model of human hepatocellular carcinoma. Huh-1 cells present higher levels of p62 compared to the immortalized HEK293 cell line. In addition, in Huh-1 cells, p62 is found phosphorylated on its Ser349. Either silencing of p62 or expression of its phosphorylation-defective mutant Ser349A, caused a decrease in cell proliferation *in vitro*, and a reduction of tumor growth *in vivo* (65). All these studies provide strong evidence to support that p62 promotes pro-tumorigenic properties, making now necessary to elucidate how p62 mechanistically promotes tumor progression.

p62 and KEAP1–NRF2 Axis

One protein positively regulated by p62 is the transcription factor NRF2. As previously mentioned, p62 facilitates KEAP1 degradation, which abolishes ubiquitylation and degradation of NRF2 (80). Thus, high levels of KEAP1 upon silencing of p62, triggers a reduction in NRF2 levels. NRF2 is consider a master regulator of the cellular antioxidant response, which regulates key target genes for cancer development and progression, such as those involved in survival, proliferation, DNA repair, and autophagy (81, 82). In particular, several evidence show the contribution of NRF2 in the properties of cancer stem cells (83–85), a subpopulation of cells present in the tumor niche involved in tumor growth, therapy resistance and metastasis (86). Ryoo and colleagues showed that high levels of NRF2 are involved in drug resistance, cell migration and invasion capacity of breast cancer stem cells (87). Importantly, silencing of p62 reduces NRF2 levels, demonstrating the regulatory role of p62 on NRF2 levels in these type of cells (87). This p62/NRF2 regulation has been also found in the glioblastoma multiforme cell line T98G, which express high levels of p62. Activation of autophagy in T98G cells leads to an increase in the levels of NRF2 (88). Moreover, and similar to what occurs in Huh-1 cells, p62 in T98G is found phosphorylated in Ser349 (88). Authors found this post-translational modification increases affinity of p62 to KEAP1 promoting its degradation by selective autophagy, with a positive impact in the stability and function of NRF2 (65). Finally, same findings have been reported in the cellular model of prostate cancer DU145, characterized by high levels of p62. Indeed, silencing of p62 in DU145 cells decrease cell proliferation, apoptosis-resistance and invasion by a mechanism related with the inactivation of the NRF2 pathway (75, 89). Altogether, these findings demonstrate the role of the axis p62/NRF2 in tumor progression in different types of cancer.

p62 and Other Possible Targets

p62 regulates several other proteins involved in tumor progression. One interesting target is the Vitamin D receptor (VDR). The VDR has a protective role in cancer due to its anti-proliferative and pro-apoptotic actions (90). In fact, VDR downregulation is associated with a poor prognosis and cancer progression (91). In this regard, high levels of p62 are correlated with a decrease in the levels of VDR in colorectal cancer (78), probably mediated by selective autophagy degradation. The authors showed that through its direct interaction, p62/VDR contributed to the pro-tumorigenic properties of two cell lines of colorectal cancer (SW480 and HCT116), promoting tumor progression *in vivo* (78). Another target of p62 is the transcription factor TWIST1, a crucial protein that facilitates epithelial mesenchymal transition (EMT) (92). Interestingly, the ubiquitin-associated domain of p62 interacts with TWIST1 to block its degradation by autophagy (92). Strikingly, overexpression of p62 in the A431 human skin cancer cell, which does not express TWIST1, is unable to increase cell migration. In contrast, when p62 is overexpressed together with TWIST1 in A431 cells, an increase in cell migration, tumor growth and metastasis is observed, proposing a functional link between p62/TWIST1 in promoting pro-tumorigenic effects *in vivo* (92). Another protein implicated in the pro-tumorigenic effects of p62 is Vimentin, a protein involved in tumor progression. Vimentin is a Type III intermediate filament that regulates cell shape, motility, and adhesion during EMT, processes implicated in cell invasion and aggressiveness in cancer cells (93, 94). In the highly metastatic MDA-MB-231 breast cancer cell line, vimentin co-immunoprepicitates with endogenous p62. Interestingly,

silencing of p62 leads to a decrease in the levels of Vimentin correlating with a reduction in the invasive capacity of these cells. Importantly, overexpression of Vimentin is sufficient to rescue this tumoral property (71). Besides, the increase phenotype in invasive capacity of MDA-MB-231 by overexpression of p62 is dependent on Vimentin levels, demonstrating Vimentin plays a crucial role in p62-mediated invasion in breast cancer cells (71). The molecular mechanism by which p62 regulates Vimentin levels remains unknown. However, and similar to the findings with TWIST1, it is possible that p62 could act by preventing Vimentin degradation. In addition, p62 is also implicated in the selective degradation of dysfunctional mitochondrial by mitophagy in acute myeloid leukemia cells (74). In fact, p62 promotes myeloid transformation, cell proliferation, leukemia development and progression of acute myeloid leukemia by a process dependent on the efficient degradation of mitochondria by mitophagy (74).

Neighbor of BRACA1 Gene1

NBR1 (neighbor of BRCA1 gene1) is an autophagy receptor with several domains including a PB1, CC1, LIRs, and UBA (95). Its PB1 domain, allows NBR1 oligomerization with either itself or p62 where these two receptors act either independently or cooperatively in the recognition of cargos for degradation (95). Similar to PB1, CC1 domain also facilitates NBR1 oligomerization. Indeed, deletion of the CC1 domain on NBR1 impairs its oligomerization and avidity to bind ubiquitin (96). Both LIRs domains can individually interact with Atg8s-proteins, where LIR1 is the most functional domain (95). Finally, the UBA domain mediates binding of NBR1 to monoubiquitin or poly-ubiquitin chains (51, 96) (**Figure 3**). Although the most common function of NBR1 is associated with its role as an autophagy receptor of autophagosomes, NBR1 can also be found associated with endosomal membranes, where it seems to mediate the delivery of certain cargos (52, 96–98). In terms of expression, NBR1 is expressed in all tissues, showing its highest expression in testis and thyroid (99).

NBR1 in Cancer Patients

Little information is known about the role of NBR1 in cancer. Data extracted from the human protein atlas (www.proteinatlas.org) showed mRNA expression of NBR1 in 17 different types of cancer with low cancer specificity (**Supplementary Figure 1**), whereas NBR1 protein levels in cancer samples displayed weak to moderate cytoplasmic expression (100). Related to cancer prognosis, it has been reported that low mRNA levels of NBR1 predict a poor clinical outcome in patients with clear cell renal carcinoma (101).

NBR1 in Migration and Metastasis

Although the clinical data available show a negative association between the NBR1 mRNA levels and the prognosis of patients with cancer, other findings suggest a positive contribution of NBR1 in the acquisition of pro-tumorigenic properties. For example, it is known that NBR1 contributes for cancer cell migration, a process finely regulated by structures called focal adhesions (FAs), a large protein complex that connects tumor cells with the extracellular matrix (ECM) through the action of integrins (102). Turnover of the FAs is essential for the migratory rate of tumor cells dependent on the assembly and disassembly of these complexes, processes that impact positively pro-tumorigenic properties (103). For instance, in a cellular model of breast cancer cell known as HRAS-transformed MCF10A cells that mimic an early stage of the tumor progression cascade, NBR1 binds ubiquitylated proteins of FAs mediating their degradation by autophagy. Indeed, reduction of NBR1 levels reduces FAs turnover with a negative impact in breast cancer cell migration (104). This effect is not observed in other breast cancer cell models, indicating some level of specificity of NBR1 function depending on the cell type and stages of the tumor progression. Accordingly, recent studies have demonstrated that NBR1 plays an important role in breast cancer metastatic progression. First, it was demonstrated that autophagy promotes growth of the primary breast cancer tumor but with a negative impact in the metastasis stage. In contrast, inhibition of autophagy showed an impairment in tumor growth but with a positive impact in metastasis (105). Moreover, it was found a robust accumulation of NBR1, suggesting that intracellular accumulation of NBR1 plays a role on metastasis (105). Interestingly, ectopic NBR1 overexpression in breast cancer cells is sufficient to promote metastatic outgrowth. Contrary, silencing of NBR1 suppresses cancer dissemination. However, the mechanism by which NBR1 promotes metastasis is still unknown. Since the effect of NBR1 on metastasis is related with inhibition of autophagy, it opens the possibility that NBR1 mediates metastasis by a non-canonical function possibly related with its role on endosome membranes. In this regard, it has been shown that NBR1 prevents the degradation of tyrosine kinase receptors, such as epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR), causing the accumulation of these cargos in endosome compartments (98), a key aspect in the control of their signaling (106–111).

NBR1 Function in Evasion of the Immune System

The immune system has the potential to recognize and eliminate tumor cells, therefore escape to the immune surveillance, which contributes to cancer progression (12). A common mechanism used by tumor cells to evade the immune system, specifically CD8+ T cells, is the impairment of the antigen presentation, which can be the result of mutations or loss of the expression of the major histocompatibility complex class I (MHC-I) (112–114). In pancreatic ductal adenocarcinoma, resistant mostly to all therapies, MHC-I is found downregulated due to the consequence of mutations in MHC-I (114). Furthermore, MHC-I is not found at the cell surface of these cells, instead it accumulates in intracellular membranes. Surprisingly, silencing of ULK1/2 complex (ULK1, FIP200 or ATG13), a protein complex implicated in the initiation of autophagosome biogenesis is sufficient to rescue the levels of MHC-I at the cell surface (114). Among all autophagy receptors, it is known that NBR1 interacts with ubiquitylated MHC-I. Moreover, silencing of NBR1 rescues the levels of MHC-I at the cell surface of

pancreatic ductal adenocarcinoma cells. Together, all these antecedents strongly indicate that distribution of MHC-I at the cell surface is controlled by NBR1 selective autophagy, highlighting NBR1 as a crucial molecule in how tumor cells evade the immune system (114).

NBR1 and Loss of Primary Cilium in Cancer

Primary cilia are non-motile microtubule-based cellular organelles present in nearly every cell that gather information about the environment, triggering a variety of cellular responses through specific intracellular signaling pathways (115). The primary cilium is dynamically regulated during the cell cycle, disappearing transitorily during cellular division (116). Importantly, loss of primary cilia has been reported in different cancer cells and tumoral tissues including pancreatic, renal, and hepatic carcinomas (117, 118). Interestingly, it has been reported that in a cellular model of cholangiocarcinoma, a type of hepatic cancer, autophagosomes are located in the primary cilia, suggesting a role of autophagy in their maintenance. Indeed, LC3 interacts with the ciliary proteins IFT88 and α-tubulin. Moreover, in comparison with others autophagy genes, NBR1 expression is found increased in intrahepatic cholangiocarcinoma tumor samples compared to normal controls. In addition, silencing of NBR1 in HuCCT1 cells, a cell line of cholangiocarcinoma, increases the size of the primary cilia (119). These antecedents suggest that NBR1 could be implicated in the degradation of ciliary component through selective autophagy, explaining the loss of the primary cilium in cholangiocarcinoma.

Nuclear Dot Protein 52 KDa

Nuclear dot protein 52 KDa [NDP52, also known as calcium binding and coiled-coil domain 2 (CALCOCO2)] is composed by the skeletal muscle and kidney-enriched inositol phosphatase carboxyl homology domain (SKICH), LC3C-specific LC3-interacting region (CLIR), Coiled-coil (CC), Galectin-8 binding region (GalBi), and ubiquitin-binding zinc finger (UBZ) domain (51, 120, 121). In mammals, *NDP52* is located on the chromosome 17 and is composed of 15 exons. The role of SKICH domain in autophagy is not yet completely understood. However, it is known that SKICH domain is responsible in the binding of NDP52 to the mitochondrial RNA poly(A) polymerase (MTPAP) in depolarized mitochondria to enhance mitophagy (122). On another hand, the CLIR domain in NDP52 is a non-canonical LIR motif that confers selective and strong binding to LC3C, with a very weak affinity to other Atg8s proteins members (120). The CC domain in NDP52 participates in its homodimerization facilitating the binding to LC3C (121, 123). The GalBi domain allows the binding of NDP52 to Galectin-8 in the context of degradation of pathogens (xenophagy) or damaged lysosomes (lysophagy), selective forms of autophagy (124, 125). Finally, the UBZ domain allows NDP52 binding to ubiquitin (mono or poly-ubiquitin) (51, 58) (**Figure 3**).

NDP52 and Its Role in Cancer Cell Survival

NDP52 has been detected in different cancer tissues with a moderated protein expression, including the majority of renal cancers. In contrast, in few cases of malignant gliomas, malignant lymphomas, skin, and lung cancers, NDP52 protein expression is almost undetected [Human protein atlas (99)]. Although the role of NDP52 in cancer is still unknown, recent evidence suggests that NDP52 could have a role in the acquisition of some pro-tumorigenic properties such as cell survival. For instance, in the cellular model of non-small cell lung cancer, cell line A549, NDP52 is found bound to LC3 in autophagosomes under basal conditions. NDP52 mediates selective degradation of the tumor necrosis factor receptor-associated factor 3 (TRAF3), a repressor of activation and nuclear translocation of RELB, an effector of non-canonical NF-κB signaling, which is usually implicated in pro-tumorigenic properties (126, 127). Interestingly, silencing of NDP52 impairs the localization of RELB into the nucleus and downregulated the expression of anti-apoptotic target genes of REL-B (128). In addition, activation and translocation of RELB due to the degradation of TRAF3 by NDP52, inhibits the transcription factor SMAD leading to a reduction in the expression of the transforming growth factor β (TGFβ), with known tumor-suppressive functions (126, 129). This inhibition promotes proliferation of A549 cells and tumor growth in animal models of non-small cell lung cancer (126).

Optineurin

Optic neuropathy inducing, also called Optineurin (OPTN), is composed by three Coiled-coil domains (CC-1, CC-2, and CC-3), a leucine zipper (LZ), LIR, ubiquitin-binding domain of ABIN proteins and NEMO (UBAN) and zinc finger (ZF) domain (130). The CC-1 domain, located in the N-terminal of OPTN binds serine/threonine TANK-binding kinase 1 (TBK1) leading to the formation of a stable OPTN-TBK1 hetero-tetramer complex. TBK1 phosphorylates the Ser172 on LIR domain of OPTN enhancing its binding to ATG8s proteins. In addition, TBK1 phosphorylates the Ser473 located on the UBAN domain leading to an increase in the binding to ubiquitin (131). The LIR domain binds to all members of ATG8s family, but compared to other autophagy receptors, the LIR domain of OPTN is the unique phosphorylated by TBK1 (51, 132). The CC-3 domain mediates the homodimerization of OPTN. Only in this form, OPTN binds, through the UBAN domain, to methionine1 (Met1)-linked linear polyubiquitin (Met1-l-polyUb) of ubiquitylated cargos in a reason of 2:1 (53, 131, 133) (**Figure 3**).

OPTN in Cancer Tumor Progression

RNA-seq data of 17 different types of cancer show that OPTN is overexpressed in pancreatic cancer, being the second most expressed autophagy receptor, after p62, in this type of cancer, and its expression correlated with a reduced survival of pancreatic ductal adenocarcinoma patients (134). Furthermore, the silencing of OPTN in different cells lines of pancreatic ductal adenocarcinoma promotes cell cycle arrest, decreases colony formation and induces apoptosis through ER stress activation (134). These antecedents indicate OPTN could play a relevant role in pancreatic ductal adenocarcinoma cells. However, it is necessary to find new cargos, which could work as negative regulatory proteins of the cell cycle.

CANCER THERAPY AND SELECTIVE AUTOPHAGY

Chemotherapy is the main strategy for cancer treatment, characterized by the use of drugs that alter and kill tumoral cells rapidly (135). These drugs include anti-mitotic agents (e.g., paclitaxel and docetaxel), topoisomerase II inhibitors (e.g., doxorubicin and epirubicin) and DNA alkylating agents (e.g., cisplatin and carboplatin) (135). Regrettably, tumor cells respond developing a variety of cellular adaptation programs that provide the ability to tolerate the cytotoxic effects of chemotherapy (135, 136). One of this responses is the activation of autophagy, a pathway that helps in the evasion of the effects of chemotherapies in tumor cells transforming them in cells resistant to chemotherapy (135–138). Indeed, it has been previously summarized the contribution of autophagy in chemoresistance in different types of tumor under different chemotherapeutic agents, proposing that autophagy inhibition is a good strategy to promote sensitization to chemotherapy (135). However, the role of autophagy receptors in chemoresistance has been poorly explored. Only recent studies have started to propose p62 as a possible target of intervention (139, 140). Cisplatin is one of the most used chemotherapeutic agent, but several studies have reported development of resistance to this chemotherapeutic agent (141). Alsamman and El-Masry showed that cisplatin promotes the increase in p62 levels in cellular models of breast, colon and ovarian cancer (139). Interestingly, treatment of these cells with cisplatin in combination with Staurosporine (natural broad-spectrum antitumor agent derived from *Streptomyces staurosporeus* (142–144) abrogates the up-regulation of p62, suggesting that Staurosporine sensitizes cancer cells to cisplatin in a p62-dependent manner (139). Similarly, Sorafenib, a multikinase inhibitor chemotherapeutic agent used in the treatment of Hepatocellular carcinoma has shown, in some cases low efficacy due to development of resistance to this chemotherapeutic agent (140). Sorafenib causes the upregulation of the KEAP1-NRF2 axis associated with an increase in the phosphorylation of p62 at Ser349 and chemoresistance (140, 145). Surprisingly, blocking interaction between KEAP1 and phospho-p62 at Ser349 seems to be sufficient to sensitize resistant cells to Sorafenib (140).

CONCLUSION AND FUTURE PERSPECTIVES

Several evidences indicate that autophagy receptors play a crucial role in cancer progression. Among all autophagy receptors identified, p62 is by far the most characterized one, currently considered a good predictor marker of the grade of malignancy in several types of cancer (71–75). Since the pro-tumoral effects of p62 are not only related with the degradation of specific cargos such as what occurs with VDR or damaged mitochondria, it opens the possibility of non-canonical roles of p62 mostly related with the stability of certain proteins like NRF2, TWIST1 and Vimentin (65, 71, 75, 80, 87). A challenge for the future to better understand the contribution of p62 during cancer progression is the identification of novel cargos of this receptor, considering specific types of cancer cells including cancer stem cells. Furthermore, it is critical to investigate these aspects studying the variety of stages during cancer progression. This type of approach could offer valuable information for the design of novel strategies in cancer treatment reducing the side effects commonly observed with current treatments. In addition to p62, recent findings highlight the role of NBR1 in cancer progression, controlling the presence of important molecules and structures implicated in pro-tumorigenic properties such as MHC-I, FAs and cilia (104, 114, 119). It is now key to decipher the regulatory mechanisms underlying their specific recognition. Similarly, it opens the possibility of NBR1 functioning as a crucial regulator of cancer signaling pathways associated with EGFR and FGFR (98). Although there is very little information about the pro-tumorigenic roles of NDP52 and OPTN, its presence in several types of cancer, and even its overexpression in the case of OPTN, make these receptors interesting targets to study during tumor progression. In this regard, since OPTN and NDP52 participate in mitophagy (125, 146, 147), it is relevant to explore the contribution of active mitophagy pathway in tumor progression and metastasis.

In conclusion, autophagy receptors are interesting molecules with validated contribution in different tumoral contexts that promotes a variety of cellular properties during cancer progression, and therefore must be considered possible targets for cancer treatment.

AUTHOR CONTRIBUTIONS

CC-T, MV-G, and PB organized the entire manuscript, wrote the draft, and revised the last version of the manuscript. Figures were designed by CC-T. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by Fondo Nacional de Desarrollo Científico y Tecnológico of Chile (FONDECYT; http://www.conicyt.cl/fondecyt), grant numbers 1190928 (MV-G) and 1171649 (PB), by ANID/AFB170005 and by Proyecto ANILLO ACT172066.

ACKNOWLEDGMENTS

We thank Fabián Díaz for his graphical illustration assistance in the schematic drawings of the figures and Lara J. Monteiro for critically reading of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.619727/full#supplementary-material

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* (2018) 68:394–424. doi: 10.3322/caac.21492
2. Pitot HC. The molecular biology of carcinogenesis. *Cancer* (1993) 72:962–70. doi: 10.1002/1097-0142(19930801)72:3+<962::AID-CNCR2820721303>3.0.CO;2-H
3. Marongiu F, Serra M, Laconi E. Development versus Evolution in Cancer Biology. *Trends Cancer* (2018) 4:342–8. doi: 10.1016/j.trecan.2018.03.007
4. Hennings H, Glick AB, Greenhalgh DA, Morgan DL, Strickland JE, Tennenbaum T, et al. Critical Aspects of Initiation, Promotion, and Progression in Multistage Epidermal Carcinogenesis. *Exp Biol Med* (1993) 202:1–8. doi: 10.3181/00379727-202-43511A
5. Oliveira PA, Colaço A, Chaves R, Guedes-Pinto H, De-La-Cruz LFP, Lopes C. Chemical carcinogenesis. *Acad Bras Cienc* (2007) 79:593–916. doi: 10.1590/s0001-37652007000400004
6. Conti CJ. Mechanisms of Tumor Progression. In: *Comprehensive Toxicology*. Kidlington, United Kingdom: Elsevier (2010). p. 335–47. doi: 10.1016/B978-0-08-046884-6.01417-2
7. Chakravarthi BVSK, Nepal S, Varambally S. Genomic and Epigenomic Alterations in Cancer. *Am J Pathol* (2016) 186:1724–35. doi: 10.1016/j.ajpath.2016.02.023
8. Harris TJR, McCormick F. The molecular pathology of cancer. *Nat Rev Clin Oncol* (2010) 7:251–65. doi: 10.1038/nrclinonc.2010.41
9. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. *Cell* (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
10. Suzuki H, Tomida A, Tsuruo T. Dephosphorylated hypoxia-inducible factor 1α as a mediator of p53-dependent apoptosis during hypoxia. *Oncogene* (2001) 20:5779–88. doi: 10.1038/sj.onc.1204742
11. Zhou X, Hao Q, Lu H. Mutant p53 in cancer therapy—the barrier or the path. *J Mol Cell Biol* (2019) 11:293–305. doi: 10.1093/jmcb/mjy072
12. Spranger S, Gajewski TF. Mechanisms of Tumor Cell–Intrinsic Immune Evasion. *Annu Rev Cancer Biol* (2018) 2:213–28. doi: 10.1146/annurev-cancerbio-030617-050606
13. Bielenberg DR, Zetter BR. The Contribution of Angiogenesis to the Process of Metastasis. *Cancer J (United States)* (2015) 21:267–73. doi: 10.1097/PPO.0000000000000138
14. Tonini T, Rossi F, Claudio PP. Molecular basis of angiogenesis and cancer. *Oncogene* (2003) 22:6549–56. doi: 10.1038/sj.onc.1206816
15. Nguyen DX, Bos PD, Massagué J. Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer* (2009) 9:274–84. doi: 10.1038/nrc2622
16. He P, Qiu K, Jia Y. Modeling of mesenchymal hybrid epithelial state and phenotypic transitions in EMT and MET processes of cancer cells. *Sci Rep* (2018) 8:14323. doi: 10.1038/s41598-018-32737-z
17. Fares J, Fares MY, Khachfe HH, Salhab HA, Fares Y. Molecular principles of metastasis: a hallmark of cancer revisited. *Signal Transduct Target Ther* (2020) 5:28. doi: 10.1038/s41392-020-0134-x
18. Martin GS. Cell signaling and cancer. *Cancer Cell* (2003) 4:167–74. doi: 10.1016/S1535-6108(03)00216-2
19. White E. The role for autophagy in cancer. *J Clin Invest* (2015) 125:42–6. doi: 10.1172/JCI73941
20. Galluzzi L, Pietrocola F, Bravo-San Pedro JM, Amaravadi RK, Baehrecke EH, Cecconi F, et al. Autophagy in malignant transformation and cancer progression. *EMBO J* (2015) 34:856–80. doi: 10.15252/embj.201490784
21. Poillet-Perez L, White E. Role of tumor and host autophagy in cancer metabolism. *Genes Dev* (2019) 33:610–9. doi: 10.1101/gad.325514.119
22. Li X, He S, Ma B. Autophagy and autophagy-related proteins in cancer. *Mol Cancer* (2020) 19:1–16. doi: 10.1186/s12943-020-1138-4
23. Xie Z, Xie Y, Xu Y, Zhou H, Xu W, Dong Q. Bafilomycin A1 inhibits autophagy and induces apoptosis in MG63 osteosarcoma cells. *Mol Med Rep* (2014) 10:1103–7. doi: 10.3892/mmr.2014.2281
24. Xiong X, Wu M, Zhang H, Li J, Lu B, Guo Y, et al. Atg5 siRNA inhibits autophagy and enhances norcantharidin-induced apoptosis in hepatocellular carcinoma. *Int J Oncol* (2015) 47:1321–8. doi: 10.3892/ijo.2015.3103
25. Lin YC, Lin JF, Wen SI, Yang SC, Tsai TF, Chen HE, et al. Chloroquine and hydroxychloroquine inhibit bladder cancer cell growth by targeting basal autophagy and enhancing apoptosis. *Kaohsiung J Med Sci* (2017) 33:215–23. doi: 10.1016/j.kjms.2017.01.004
26. Dong Y, Wu Y, Zhao GL, Ye ZY, Xing CG, Yang XD. Inhibition of autophagy by 3-MA promotes hypoxia-induced apoptosis in human colorectal cancer cells. *Eur Rev Med Pharmacol Sci* (2019) 23:1047–54. doi: 10.26355/eurrev_201902_16992
27. Scherr AL, Jassowicz A, Pató A, Elssner C, Ismail L, Schmitt N, et al. Knockdown of Atg7 induces nuclear-LC3 dependent apoptosis and augments chemotherapy in colorectal cancer cells. *Int J Mol Sci* (2020) 21:1099. doi: 10.3390/ijms21031099
28. Fulda S. Autophagy in Cancer Therapy. *Front Oncol* (2017) 7:128. doi: 10.3389/fonc.2017.00128
29. Hwang M, Jun DW, Kang EH, Yoon K-A, Cheong H, Kim Y-H, et al. EI24, as a Component of Autophagy, Is Involved in Pancreatic Cell Proliferation. *Front Oncol* (2019) 9:652:652. doi: 10.3389/fonc.2019.00652
30. Dikic I, Elazar Z. Mechanism and medical implications of mammalian autophagy. *Nat Rev Mol Cell Biol* (2018) 19:349–64. doi: 10.1038/s41580-018-0003-4
31. Wollert T. Autophagy. *Curr Biol* (2019) 29:R671–7. doi: 10.1016/j.cub.2019.06.014
32. Gatica D, Lahiri V, Klionsky DJ. Cargo recognition and degradation by selective autophagy. *Nat Cell Biol* (2018) 20:233–42. doi: 10.1038/s41556-018-0037-z
33. Levine B, Kroemer G. Biological Functions of Autophagy Genes: A Disease Perspective. *Cell* (2019) 176:11–42. doi: 10.1016/j.cell.2018.09.048
34. Xie Z, Klionsky DJ. Autophagosome formation: Core machinery and adaptations. *Nat Cell Biol* (2007) 9:1102–9. doi: 10.1038/ncb1007-1102
35. Nishimura T, Tamura N, Kono N, Shimanaka Y, Arai H, Yamamoto H, et al. Autophagosome formation is initiated at phosphatidylinositol synthase-enriched ER subdomains. *EMBO J* (2017) 36:1719–35. doi: 10.15252/embj.201695189
36. Nath S, Dancourt J, Shteyn V, Puente G, Fong WM, Nag S, et al. Lipidation of the LC3/GABARAP family of autophagy proteins relies on a membrane-curvature-sensing domain in Atg3. *Nat Cell Biol* (2014) 16:415–24. doi: 10.1038/ncb2940
37. Carlsson SR, Simonsen A. Membrane dynamics in autophagosome biogenesis. *J Cell Sci* (2015) 128:193–205. doi: 10.1242/jcs.141036
38. Kabeya Y, Mizushima N, Yamamoto A, Oshitani-Okamoto S, Ohsumi Y, Yoshimori T. LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. *J Cell Sci* (2004) 117:2805–12. doi: 10.1242/jcs.01131
39. Goold R, McKinnon C, Rabbanian S, Collinge J, Schiavo G, Tabrizi S. Alternative fates of newly formed PrPSc upon prion conversion on the plasma membrane. *J Cell Sci* (2013) 126:3237–47. doi: 10.1242/jcs.126128
40. Johansen T, Lamark T. Selective Autophagy: ATG8 Family Proteins, LIR Motifs and Cargo Receptors. *J Mol Biol* (2020) 432:80–103. doi: 10.1016/j.jmb.2019.07.016
41. Mancias JD, Kimmelman AC. Mechanisms of Selective Autophagy in Normal Physiology and Cancer. *J Mol Biol* (2016) 428:1659–80. doi: 10.1016/j.jmb.2016.02.027
42. Shaid S, Brandts CH, Serve H, Dikic I. Ubiquitination and selective autophagy. *Cell Death Differ* (2013) 20:21–30. doi: 10.1038/cdd.2012.72
43. Stolz A, Ernst A, Dikic I. Cargo recognition and trafficking in selective autophagy. *Nat Cell Biol* (2014) 16:495–501. doi: 10.1038/ncb2979
44. Khaminets A, Heinrich T, Mari M, Huebner AK, Akutsu M, Grumati P, et al. Regulation of endoplasmic reticulum turnover by FAM134B-mediated selective autophagy. *Nature* (2015) 522:359–62. doi: 10.1038/nature14498
45. Fumagalli F, Noack J, Bergmann TJ, Presmanes EC, Pisoni GB, Fasana E, et al. Translocon component Sec62 acts in endoplasmic reticulum turnover during stress recovery. *Nat Cell Biol* (2016) 18:1173–84. doi: 10.1038/ncb3423
46. Grumati P, Morozzi G, Hölper S, Mari M, Harwardt M-LI, Yan R, et al. Full length RTN3 regulates turnover of tubular endoplasmic reticulum via selective autophagy. *Elife* (2017) 6:e25555. doi: 10.7554/eLife.25555
47. Lahiri V, Klionsky DJ. CCPG1 is a noncanonical autophagy cargo receptor essential for reticulophagy and pancreatic ER proteostasis. *Autophagy* (2018) 14:1107–9. doi: 10.1080/15548627.2018.1467199

48. Chino H, Hatta T, Natsume T, Mizushima N. Intrinsically Disordered Protein TEX264 Mediates ER-phagy. *Mol Cell* (2019) 74:909–21.e6. doi: 10.1016/j.molcel.2019.03.033
49. Tanida I, Minematsu-Ikeguchi N, Ueno T, Kominami E. Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. *Autophagy* (2005) 1:84–91. doi: 10.4161/auto.1.2.1697
50. Bjørkøy G, Lamark T, Brech A, Outzen H, Perander M, Øvervatn A, et al. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol* (2005) 171:603–14. doi: 10.1083/jcb.200507002
51. Kim B-W, Kwon DH, Song HK. Structure biology of selective autophagy receptors. *BMB Rep* (2016) 49:73–80. doi: 10.5483/BMBRep.2016.49.2.265
52. Mejlvang J, Olsvik H, Svenning S, Bruun JA, Abudu YP, Larsen KB, et al. Starvation induces rapid degradation of selective autophagy receptors by endosomal microautophagy. *J Cell Biol* (2018) 217:3640–55. doi: 10.1083/jcb.201711002
53. Dikic I, Wakatsuki S, Walters KJ. Ubiquitin-binding domains - from structures to functions. *Nat Rev Mol Cell Biol* (2009) 10:659–71. doi: 10.1038/nrm2767
54. Nicot AS, Verso FL, Ratti F, Pilot-Storck F, Streichenberger N, Sandri M, et al. Phosphorylation of NBR1 by GSK3 modulates protein aggregation. *Autophagy* (2014) 10:1036–53. doi: 10.4161/auto.28479
55. Lim J, Lachenmayer ML, Wu S, Liu W, Kundu M, Wang R, et al. Proteotoxic Stress Induces Phosphorylation of p62/SQSTM1 by ULK1 to Regulate Selective Autophagic Clearance of Protein Aggregates. *PloS Genet* (2015) 11:e1004987. doi: 10.1371/journal.pgen.1004987
56. Richter B, Sliter DA, Herhaus L, Stolz A, Wang C, Beli P, et al. Phosphorylation of OPTN by TBK1 enhances its binding to Ub chains and promotes selective autophagy of damaged mitochondria. *Proc Natl Acad Sci USA* (2016) 113:4039–44. doi: 10.1073/pnas.1523926113
57. You Z, Jiang W-X, Qin L-Y, Gong Z, Wan W, Li J, et al. Requirement for p62 acetylation in the aggregation of ubiquitylated proteins under nutrient stress. *Nat Commun* (2019) 10:5792. doi: 10.1038/s41467-019-13718-w
58. Husnjak K, Dikic I. Ubiquitin-Binding Proteins: Decoders of Ubiquitin-Mediated Cellular Functions. *Annu Rev Biochem* (2012) 81:291–322. doi: 10.1146/annurev-biochem-051810-094654
59. Liu WJ, Ye L, Huang WF, Guo LJ, Xu ZG, Wu HL, et al. p62 links the autophagy pathway and the ubiqutin–proteasome system upon ubiquitinated protein degradation. *Cell Mol Biol Lett* (2016) 21:29. doi: 10.1186/s11658-016-0031-z
60. Kwon DH, Park OH, Kim L, Jung YO, Park Y, Jeong H, et al. Insights into degradation mechanism of N-end rule substrates by p62/SQSTM1 autophagy adapter. *Nat Commun* (2018) 9:1–13. doi: 10.1038/s41467-018-05825-x
61. Zhang Y, Mun SR, Linares JF, Ahn J, Towers CG, Ji CH, et al. ZZ-dependent regulation of p62/SQSTM1 in autophagy. *Nat Commun* (2018) 9:4373. doi: 10.1038/s41467-018-06878-8
62. Yoo YD, Mun SR, Ji CH, Sung KW, Kang KY, Heo AJ, et al. N-terminal arginylation generates a bimodal degron that modulates autophagic proteolysis. *Proc Natl Acad Sci USA* (2018) 115:E2716–24. doi: 10.1073/pnas.1719110115
63. Ji CH, Kim HY, Heo AJ, Lee SH, Lee MJ, Bin KS, et al. The N-Degron Pathway Mediates ER-phagy. *Mol Cell* (2019) 75:1–15. doi: 10.1016/j.molcel.2019.06.028
64. Lee YJ, Chou TF, Pittman SK, Keith AL, Razani B, Weihl CC. Keap1/Cullin3 Modulates p62/SQSTM1 Activity via UBA Domain Ubiquitination. *Cell Rep* (2017) 19:188–202. doi: 10.1016/j.celrep.2017.03.030
65. Ichimura Y, Waguri S, Sou YS, Kageyama S, Hasegawa J, Ishimura R, et al. Phosphorylation of p62 Activates the Keap1-Nrf2 Pathway during Selective Autophagy. *Mol Cell* (2013) 51:618–31. doi: 10.1016/j.molcel.2013.08.003
66. Kachaner D, Filipe J, Laplantine E, Bauch A, Bennett KL, Superti-Furga G, et al. Plk1-Dependent Phosphorylation of Optineurin Provides a Negative Feedback Mechanism for Mitotic Progression. *Mol Cell* (2012) 45:553–66. doi: 10.1016/j.molcel.2011.12.030
67. Linares JF, Duran A, Reina-Campos M, Aza-Blanc P, Campos A, Moscat J, et al. Amino Acid Activation of mTORC1 by a PB1-Domain-Driven Kinase Complex Cascade. *Cell Rep* (2015) 12:1339–52. doi: 10.1016/j.celrep.2015.07.045
68. Pan JA, Sun Y, Jiang YP, Bott AJ, Jaber N, Dou Z, et al. TRIM21 Ubiquitylates SQSTM1/p62 and Suppresses Protein Sequestration to Regulate Redox Homeostasis. *Mol Cell* (2016) 61:720–33. doi: 10.1016/j.molcel.2016.02.007
69. Inoue D, Suzuki T, Mitsuishi Y, Miki Y, Suzuki S, Sugawara S, et al. Accumulation of p62/SQSTM1 is associated with poor prognosis in patients with lung adenocarcinoma. *Cancer Sci* (2012) 103:760–6. doi: 10.1111/j.1349-7006.2012.02216.x
70. Schläfli AM , Adams O, Galván JA, Gugger M, Savic S, Bubendorf L, et al. Prognostic value of the autophagy markers LC3 and p62/SQSTM1 in early-stage non-small cell lung cancer. *Oncotarget* (2016) 7:39544–55. doi: 10.18632/oncotarget.9647
71. Li SS, Xu LZ, Zhou W, Yao S, Wang CL, Xia JL, et al. p62/SQSTM1 interacts with vimentin to enhance breast cancer metastasis. *Carcinogenesis* (2017) 38:1092–103. doi: 10.1093/carcin/bgx099
72. Nakayama S, Karasawa H, Suzuki T, Yabuuchi S, Takagi K, Aizawa T, et al. p62/sequestosome 1 in human colorectal carcinoma as a potent prognostic predictor associated with cell proliferation. *Cancer Med* (2017) 6:1264–74. doi: 10.1002/cam4.1093
73. Lu Y, Wang Q, Zhou Y, Sun L, Hu B, Xue H, et al. Overexpression of p62 is associated with poor prognosis and aggressive phenotypes in osteosarcoma. *Oncol Lett* (2018) 15:9889–95. doi: 10.3892/ol.2018.8579
74. Nguyen D, Shaid S, Vakhrusheva O, Koschade SE, Klann K, Thölken M, et al. Loss of the selective autophagy receptor p62 impairs murine myeloid leukemia progression and mitophagy. *Blood* (2019) 133:168–79. doi: 10.1182/blood-2018-02-833475
75. Jiang G, Liang X, Huang Y, Lan Z, Zhang Z, Su Z, et al. P62 promotes proliferation, apoptosis–resistance and invasion of prostate cancer cells through the Keap1/Nrf2/ARE axis. *Oncol Rep* (2020) 43:1547–57. doi: 10.3892/or.2020.7527
76. Nihira K, Miki Y, Ono K, Suzuki T, Sasano H. An inhibition of p62/SQSTM1 caused autophagic cell death of several human carcinoma cells. *Cancer Sci* (2014) 105:568–75. doi: 10.1111/cas.12396
77. Xu L, Xu F, Kong Q, Yang T, Tan D, Zhang X, et al. Inhibition of p62/SQSTM1 sensitizes small-cell lung cancer cells to cisplatin-induced cytotoxicity by targeting NEDD9 expression. *Mol Carcinog* (2020) 59:967–79. doi: 10.1002/mc.23215
78. Zhang J, Yang S, Xu B, Wang T, Zheng Y, Liu F, et al. p62 functions as an oncogene in colorectal cancer through inhibiting apoptosis and promoting cell proliferation by interacting with the vitamin D receptor. *Cell Prolif* (2019) 52:1–13. doi: 10.1111/cpr.12585
79. Lauzier A, Normandeau-Guimond J, Vaillancourt-Lavigueur V, Boivin V, Charbonneau M, Rivard N, et al. Colorectal cancer cells respond differentially to autophagy inhibition in vivo. *Sci Rep* (2019) 9:1–16. doi: 10.1038/s41598-019-47659-7
80. Katsuragi Y, Ichimura Y, Komatsu M. Regulation of the Keap1–Nrf2 pathway by p62/SQSTM1. *Curr Opin Toxicol* (2016) 1:54–61. doi: 10.1016/j.cotox.2016.09.005
81. Rojo de la Vega M, Chapman E, Zhang DD. NRF2 and the Hallmarks of Cancer. *Cancer Cell* (2018) 34:21–43. doi: 10.1016/j.ccell.2018.03.022
82. Zimta A-A, Cenariu D, Irimie A, Magdo L, Nabavi SM, Atanasov AG, et al. The Role of Nrf2 Activity in Cancer Development and Progression. *Cancers (Basel)* (2019) 11:1755. doi: 10.3390/cancers11111755
83. Zhu J, Wang H, Sun Q, Ji X, Zhu L, Cong Z, et al. Nrf2 is required to maintain the self-renewal of glioma stem cells. *BMC Cancer* (2013) 13:1–11. doi: 10.1186/1471-2407-13-380
84. Ryoo I, Lee S, Kwak M-K. Redox Modulating NRF2: A Potential Mediator of Cancer Stem Cell Resistance. *Oxid Med Cell Longev* (2016) 2016:1–14. doi: 10.1155/2016/2428153
85. Kim D, Choi B, Ryoo I, Kwak M-K. High NRF2 level mediates cancer stem cell-like properties of aldehyde dehydrogenase (ALDH)-high ovarian cancer cells: inhibitory role of all-trans retinoic acid in ALDH/NRF2 signaling. *Cell Death Dis* (2018) 9:896. doi: 10.1038/s41419-018-0903-4
86. Prager BC, Xie Q, Bao S, Rich JN. Cancer Stem Cells: The Architects of the Tumor Ecosystem. *Cell Stem Cell* (2019) 24:41–53. doi: 10.1016/j.stem.2018.12.009
87. Ryoo I-g, Choi B-h, Ku SK, Kwak MK. High CD44 expression mediates p62-associated NFE2L2/NRF2 activation in breast cancer stem cell-like cells: Implications for cancer stem cell resistance. *Redox Biol* (2018) 17:246–58. doi: 10.1016/j.redox.2018.04.015

88. Pölönen P, Jawahar Deen A, Leinonen HM, Jyrkkänen HK, Kuosmanen S, Mononen M, et al. Nrf2 and SQSTM1/p62 jointly contribute to mesenchymal transition and invasion in glioblastoma. *Oncogene* (2019) 38:7473–90. doi: 10.1038/s41388-019-0956-6
89. Jiang X, Huang Y, Liang X, Jiang F, He Y, Li T, et al. Metastatic prostate cancer-associated P62 inhibits autophagy flux and promotes epithelial to mesenchymal transition by sustaining the level of HDAC6. *Prostate* (2018) 78:426–34. doi: 10.1002/pros.23487
90. Campbell MJ, Trump DL. Vitamin D Receptor Signaling and Cancer. *Endocrinol Metab Clin North Am* (2017) 46:1009–38. doi: 10.1016/j.ecl.2017.07.007
91. Kouchi Z, Fujiwara Y, Yamaguchi H, Nakamura Y, Fukami K. Phosphatidylinositol 5-phosphate 4-kinase type II beta is required for vitamin D receptor-dependent E-cadherin expression in SW480 cells. *Biochem Biophys Res Commun* (2011) 408:523–9. doi: 10.1016/j.bbrc.2011.04.045
92. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* (2004) 117:927–39. doi: 10.1016/j.cell.2004.06.006
93. Elzamly S, Badri N, Padilla O, Dwivedi AK, Alvarado LA, Hamilton M, et al. Epithelial-Mesenchymal Transition Markers in Breast Cancer and Pathological Responseafter Neoadjuvant Chemotherapy. *Breast Cancer Basic Clin Res* (2018) 12:117822341878807. doi: 10.1177/1178223418788074
94. Brabletz T, Kalluri R, Nieto MA, Weinberg RA. EMT in cancer. *Nat Rev Cancer* (2018) 18:128–34. doi: 10.1038/nrc.2017.118
95. Kirkin V, Lamark T, Johansen T, Dikic I. NBR1 cooperates with p62 in selective autophagy of ubiquitinated targets. *Autophagy* (2009) 5:732–3. doi: 10.4161/auto.5.5.8566
96. Kirkin V, Lamark T, Sou YS, Bjørkøy G, Nunn JL, Bruun JA, et al. A Role for NBR1 in Autophagosomal Degradation of Ubiquitinated Substrates. *Mol Cell* (2009) 33:505–16. doi: 10.1016/j.molcel.2009.01.020
97. Mardakheh FK, Yekezare M, Machesky LM, Heath JK. Spred2 interaction with the late endosomal protein NBR1 down-regulates fibroblast growth factor receptor signaling. *J Cell Biol* (2009) 187:265–77. doi: 10.1083/jcb.200905118
98. Mardakheh FK, Auciello G, Dafforn TR, Rappoport JZ, Heath JK. Nbr1 Is a Novel Inhibitor of Ligand-Mediated Receptor Tyrosine Kinase Degradation. *Mol Cell Biol* (2010) 30:5672–85. doi: 10.1128/mcb.00878-10
99. Fagerberg L, Hallstrom BM, Oksvold P, Kampf C, Djureinovic D, Odeberg J, et al. Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. *Mol Cell Proteomics* (2014) 13:397–406. doi: 10.1074/mcp.M113.035600
100. Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Tissue-based map of the human proteome. *Sci (80-)* (2015) 347:1260419–1260419. doi: 10.1126/science.1260419
101. Xiao W, Xiong Z, Yuan C, Bao L, Liu D, Yang X, et al. Low neighbor of Brca1 gene expression predicts poor clinical outcome and resistence of sunitinib in clear cell renal cell carcinoma. *Oncotarget* (2017) 8:94819–33. doi: 10.18632/oncotarget.21999
102. Janiszewska M, Primi MC, Izard T. Cell adhesion in cancer: Beyond the migration of single cells. *J Biol Chem* (2020) 295:2495–505. doi: 10.1074/jbc.REV119.007759
103. Nagano M, Hoshino D, Koshikawa N, Akizawa T, Seiki M. Turnover of Focal Adhesions and Cancer Cell Migration. *Int J Cell Biol* (2012) 2012:1–10. doi: 10.1155/2012/310616
104. Kenific CM, Stehbens SJ, Goldsmith J, Leidal AM, Faure N, Ye J, et al. NBR1 enables autophagy-dependent focal adhesion turnover. *J Cell Biol* (2016) 212:577–90. doi: 10.1083/jcb.201503075
105. Marsh T, Kenific CM, Suresh D, Gonzalez H, Shamir ER, Mei W, et al. Autophagic Degradation of NBR1 Restricts Metastatic Outgrowth during Mammary Tumor Progression. *Dev Cell* (2020) 52:591–604.e6. doi: 10.1016/j.devcel.2020.01.025
106. Burke P, Schooler K, Wiley HS. Regulation of epidermal growth factor receptor signaling by endocytosis and intracellular trafficking. *Mol Biol Cell* (2001) 12:1897–910. doi: 10.1091/mbc.12.6.1897
107. Leibl S, Zigeuner R, Hutterer G, Chromecki T, Rehak P, Langner C. EGFR expression in urothelial carcinoma of the upper urinary tract is associated with disease progression and metaplastic morphology. *APMIS* (2008) 116:27–32. doi: 10.1111/j.1600-0463.2008.00859.x
108. Villaseñor R, Kalaidzidis Y, Zerial M. Signal processing by the endosomal system. *Curr Opin Cell Biol* (2016) 39:53–60. doi: 10.1016/j.ceb.2016.02.002
109. Day KC, Hiles GL, Kozminsky M, Dawsey SJ, Paul A, Broses LJ, et al. HER2 and EGFR overexpression support metastatic progression of prostate cancer to bone. *Cancer Res* (2017) 77:74–85. doi: 10.1158/0008-5472.CAN-16-1656
110. Lee JY, Kim JM, Jeong DS, Kim MH. Transcriptional activation of EGFR by HOXB5 and its role in breast cancer cell invasion. *Biochem Biophys Res Commun* (2018) 503:2924–30. doi: 10.1016/j.bbrc.2018.08.071
111. Liang K-H, Tso H-C, Hung S-H, Kuan I-I, Lai J-K, Ke F-Y, et al. Extracellular domain of EpCAM enhances tumor progression through EGFR signaling in colon cancer cells. *Cancer Lett* (2018) 433:165–75. doi: 10.1016/j.canlet.2018.06.040
112. Axelrod ML, Cook RS, Johnson DB, Balko JM. Biological Consequences of MHC-II Expression by Tumor Cells in Cancer. *Clin Cancer Res* (2019) 25:2392–402. doi: 10.1158/1078-0432.CCR-18-3200
113. Cornel AM, Mimpen IL, Nierkens S. MHC Class I Downregulation in Cancer: Underlying Mechanisms and Potential Targets for Cancer Immunotherapy. *Cancers (Basel)* (2020) 12:1760. doi: 10.3390/cancers12071760
114. Yamamoto K, Venida A, Yano J, Biancur DE, Kakiuchi M, Gupta S, et al. Autophagy promotes immune evasion of pancreatic cancer by degrading MHC-I. *Nature* (2020) 581:100–5. doi: 10.1038/s41586-020-2229-5
115. Wheway G, Nazlamova L, Hancock JT. Signaling through the Primary Cilium. *Front Cell Dev Biol* (2018) 6:8:8. doi: 10.3389/fcell.2018.00008
116. Liu H, Kiseleva AA, Golemis EA. Ciliary signalling in cancer. *Nat Rev Cancer* (2018) 18:511–24. doi: 10.1038/s41568-018-0023-6
117. Basten SG, Willekers S, Vermaat JSP, Slaats GGG, Voest EE, van Diest PJ, et al. Reduced cilia frequencies in human renal cell carcinomas versus neighboring parenchymal tissue. *Cilia* (2013) 2:2. doi: 10.1186/2046-2530-2-2
118. Gradilone SA, Pisarello MJ, LaRusso NF. Primary Cilia in Tumor Biology: The Primary Cilium as a Therapeutic Target in Cholangiocarcinoma. *Curr Drug Targets* (2015) 18:958–63. doi: 10.2174/1389450116666150223162737
119. Peixoto E, Jin S, Thelen K, Biswas A, Richard S, Morleo M, et al. HDAC6-dependent Ciliophagy Is Involved in Ciliary Loss and Cholangiocarcinoma Growth in Human Cells and Murine Models. *Am J Physiol Liver Physiol* (2020) 318:G1022–33. doi: 10.1016/j.solener.2019.02.027
120. von Muhlinen N, Akutsu M, Ravenhill BJ, Foeglein Á, Bloor S, Rutherford TJ, et al. LC3C, Bound Selectively by a Noncanonical LIR Motif in NDP52, Is Required for Antibacterial Autophagy. *Mol Cell* (2012) 48:329–42. doi: 10.1016/j.molcel.2012.08.024
121. Kim BW, Hong SB, Kim JH, Kwon DH, Song HK. Structural basis for recognition of autophagic receptor NDP52 by the sugar receptor galectin-8. *Nat Commun* (2013) 4:1–8. doi: 10.1038/ncomms2606
122. Furuya N, Kakuta S, Sumiyoshi K, Ando M, Nonaka R, Suzuki A, et al. NDP52 interacts with mitochondrial RNA poly(A) polymerase to promote mitophagy. *EMBO Rep* (2018) 19:1–18. doi: 10.15252/embr.201846363
123. Kim BW, Jung YO, Kim MK, Kwon DH, Park SH, Kim JH, et al. ACCORD: An assessment tool to determine the orientation of homodimeric coiled-coils. *Sci Rep* (2017) 7:1–11. doi: 10.1038/srep43318
124. Thurston TLM, Wandel MP, von Muhlinen N, Foeglein Á, Randow F. Galectin 8 targets damaged vesicles for autophagy to defend cells against bacterial invasion. *Nature* (2012) 482:414–8. doi: 10.1038/nature10744
125. Anding AL, Baehrecke EH. Cleaning House: Selective Autophagy of Organelles. *Dev Cell* (2017) 41:10–22. doi: 10.1016/j.devcel.2017.02.016
126. Newman AC, Kemp AJ, Drabsch Y, Behrends C, Wilkinson S. Autophagy acts through TRAF3 and RELB to regulate gene expression via antagonism of SMAD proteins. *Nat Commun* (2017) 8:1–15. doi: 10.1038/s41467-017-00859-z
127. Tegowski M, Baldwin A. Noncanonical NF-κB in Cancer. *Biomedicines* (2018) 6:66. doi: 10.3390/biomedicines6020066
128. Newman AC, Scholefield CL, Kemp AJ, Newman M, McIver EG, Kamal A, et al. TBK1 Kinase Addiction in Lung Cancer Cells Is Mediated via Autophagy of Tax1bp1/Ndp52 and Non-Canonical NF-κB Signalling. *PloS One* (2012) 7:e50672. doi: 10.1371/journal.pone.0050672
129. Massagué J. TGFβ in Cancer. *Cell* (2008) 134:215–30. doi: 10.1016/j.cell.2008.07.001
130. Ryan TA, Tumbarello DA. Optineurin: A coordinator of membrane-associated cargo trafficking and autophagy. *Front Immunol* (2018) 9:1024. doi: 10.3389/fimmu.2018.01024
131. Li F, Xu D, Wang Y, Zhou Z, Liu J, Hu S, et al. Structural insights into the ubiquitin recognition by OPTN (optineurin) and its regulation by TBK1-

mediated phosphorylation. *Autophagy* (2018) 14:66–79. doi: 10.1080/15548627.2017.1391970
132. Ying H, Yue BYJT. Cellular and Molecular Biology of Optineurin. In: *International Review of Cell and Molecular Biology*. Amsterdam, Netherlands: Elsevier Inc. (2012) p. 223–58. doi: 10.1016/B978-0-12-394305-7.00005-7
133. Komander D, Rape M. The Ubiquitin Code. *Annu Rev Biochem* (2012) 81:203–29. doi: 10.1146/annurev-biochem-060310-170328
134. Ali DM, Ansari SS, Zepp M, Knapp-Mohammady M, Berger MR. Optineurin downregulation induces endoplasmic reticulum stress, chaperone-mediated autophagy, and apoptosis in pancreatic cancer cells. *Cell Death Discovery* (2019) 5:128. doi: 10.1038/s41420-019-0206-2
135. Ho CJ, Gorski SM. Molecular Mechanisms Underlying Autophagy-Mediated Treatment Resistance in Cancer. *Cancers (Basel)* (2019) 11:1775. doi: 10.3390/cancers11111775
136. Zheng HC. The molecular mechanisms of chemoresistance in cancers. *Oncotarget* (2017) 8:59950–64. doi: 10.18632/oncotarget.19048
137. Bao L, Jaramillo MC, Zhang Z, Zheng Y, Yao M, Zhang DD, et al. Induction of autophagy contributes to cisplatin resistance in human ovarian cancer cells. *Mol Med Rep* (2015) 11:91–8. doi: 10.3892/mmr.2014.2671
138. Alessia Battista R, Resnati M, Facchi C, Ruggieri E, Cremasco F, Paradiso F, et al. Autophagy mediates epithelial cancer chemoresistance by reducing p62/SQSTM1 accumulation. *PloS One* (2018) 13:1–18. doi: 10.1371/journal.pone.0201621
139. Alsamman K. El–Masry O. Staurosporine alleviates cisplatin chemoresistance in human cancer cell models by suppressing the induction of SQSTM1/p62. *Oncol Rep* (2018) 40:2157–62. doi: 10.3892/or.2018.6615
140. Yasuda D, Ohe T, Takahashi K, Imamura R, Kojima H, Okabe T, et al. Inhibitors of the protein–protein interaction between phosphorylated p62 and Keap1 attenuate chemoresistance in a human hepatocellular carcinoma cell line. *Free Radic Res* (2020), 1–13. doi: 10.1080/10715762.2020.1732955
141. Galluzzi L, Senovilla L, Vitale I, Michels J, Martins I, Kepp O, et al. Molecular mechanisms of cisplatin resistance. *Oncogene* (2012) 31:1869–83. doi: 10.1038/onc.2011.384
142. Xue LY, Chiu SM, Oleinick NL. Staurosporine-induced death of MCF-7 human breast cancer cells: A distinction between caspase-3-dependent steps of apoptosis and the critical lethal lesions. *Exp Cell Res* (2003) 283:135–45. doi: 10.1016/S0014-4827(02)00032-0
143. Wahba HA, El-Hadaad HA. Current approaches in treatment of triple-negative breast cancer. *Cancer Biol Med* (2015) 12:106–16. doi: 10.7497/j.issn.2095-3941.2015.0030
144. Del Solar V, Lizardo DY, Li N, Hurst JJ, Brais CJ, Atilla-Gokcumen GE. Differential Regulation of Specific Sphingolipids in Colon Cancer Cells during Staurosporine-Induced Apoptosis. *Chem Biol* (2015) 22:1662–70. doi: 10.1016/j.chembiol.2015.11.004
145. Saito T, Ichimura Y, Taguchi K, Suzuki T, Mizushima T, Takagi K, et al. P62/Sqstm1 promotes malignancy of HCV-positive hepatocellular carcinoma through Nrf2-dependent metabolic reprogramming. *Nat Commun* (2016) 7:1–16. doi: 10.1038/ncomms12030
146. Weil R, Laplantine E, Curic S, Génin P. Role of optineurin in the mitochondrial dysfunction: Potential implications in neurodegenerative diseases and cancer. *Front Immunol* (2018) 9:1243. doi: 10.3389/fimmu.2018.01243
147. Macleod KF. Mitophagy and Mitochondrial Dysfunction in Cancer. *Annu Rev Cancer Biol* (2020) 4:41–60. doi: 10.1146/annurev-cancerbio-030419-033405

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Cerda-Troncoso, Varas-Godoy and Burgos. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Oncology

REVIEW
published: 12 February 2021
doi: 10.3389/fonc.2020.614677

Chaperone Mediated Autophagy Substrates and Components in Cancer

Javiera Rios[1†], *Alvaro Sequeida*[1†], *Amelina Albornoz*[2,3] *and Mauricio Budini*[1,4*]

[1] Molecular and Cellular Pathology Laboratory, Dentistry Faculty, Institute in Dentistry Sciences, University of Chile, Santiago, Chile, [2] Fundación Ciencia & Vida, Santiago, Chile, [3] San Sebastian University, Santiago, Chile, [4] Autophagy Research Center (ARC), Santiago, Chile

Chaperone-mediated autophagy (CMA) represents a specific way of lysosomal protein degradation and contrary to macro and microautophagy is independent of vesicles formation. The role of CMA in different physiopathological processes has been studied for several years. In cancer, alterations of the CMA principal components, Hsc70 and Lamp2A protein and mRNA levels, have been described in malignant cells. However, changes in the expression levels of these CMA components are not always associated with changes in CMA activity and their biological significance must be carefully interpreted case by case. The objective of this review is to discuss whether altering the CMA activity, CMA substrates or CMA components is accurate to avoid cancer progression. In particular, this review will discuss about the evidences in which alterations CMA components Lamp2A and Hsc70 are associated or not with changes in CMA activity in different cancer types. This analysis will help to better understand the role of CMA activity in cancer and to elucidate whether CMA can be considered as target for therapeutics. Further, it will help to define whether the attention of the investigation should be focused on Lamp2A and Hsc70 because they can have an independent role in cancer progression beyond of their participation in altered CMA activity.

Keywords: CMA, oncogenic protein, tumor suppressor, autophagy, cancer progression

OPEN ACCESS

Edited by:
Daniel Hector Grasso, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

Reviewed by:
Srikanta Dash, Tulane University, United States
Henrique Girao, University of Coimbra, Portugal

***Correspondence:**
Mauricio Budini
mbudini@u.uchile.cl

[†]*These authors have contributed equally to this work*

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *06 October 2020*
Accepted: *14 December 2020*
Published: *12 February 2021*

Citation:
Rios J, Sequeida A, Albornoz A and Budini M (2021) Chaperone Mediated Autophagy Substrates and Components in Cancer. Front. Oncol. 10:614677. doi: 10.3389/fonc.2020.614677

AUTOPHAGY

Autophagy is a lysosomal dependent cellular pathway that mediates the degradation of organelles, protein aggregates and specific proteins, and is essential for cell survival, development and homeostasis (1). There are three main routes where different cargos arrive to the lysosomes (**Figure 1**): i) Macroautophagy (MA) the cargo is trapped in double membrane vesicles

Abbreviations: ATG-5, Autophagy-related gene 5; CMA, Chaperone-Mediated Autophagy; CRTC1, CREB-regulated transcription coactivator 1; EF1, Elongation Factor 1; GFAP, Glial Fibrillary acidic protein; GKN2, Gastrokine 2; GLPR1, Glioma pathogenesis-related protein 1; HIF-1, Hypoxia-inducible factor 1; HKII, Hexokinase II; Hsc70, Heat Shock cognate 70 KDa; HSD17B4, Hydroxysteroid 17-Beta Dehydrogenase 4; Hsp90, Heat Shock protein 90 Kda; IGF-IR, Insulin-like growth factor-1; Lamp2A, Lysosomal-associated membrane protein type 2A; Lys-HSc70, Lysosomal Heat Shock Cognate 70 KDa; MORC2, MORC family CW-type zinc finger 2; NSCLC, Non-Small-Cells Lung Cancer; PHLPP1, PH domain leucine-rich repeat-containing protein phosphatase 1; PRDX1, Peroxiredoxin 1; RND3, Rho Family GTPase 3; SNX-10, Sorting Nexin 10; TAM, Tumor associated macrophages; TFEB, Transcription Factor EB.

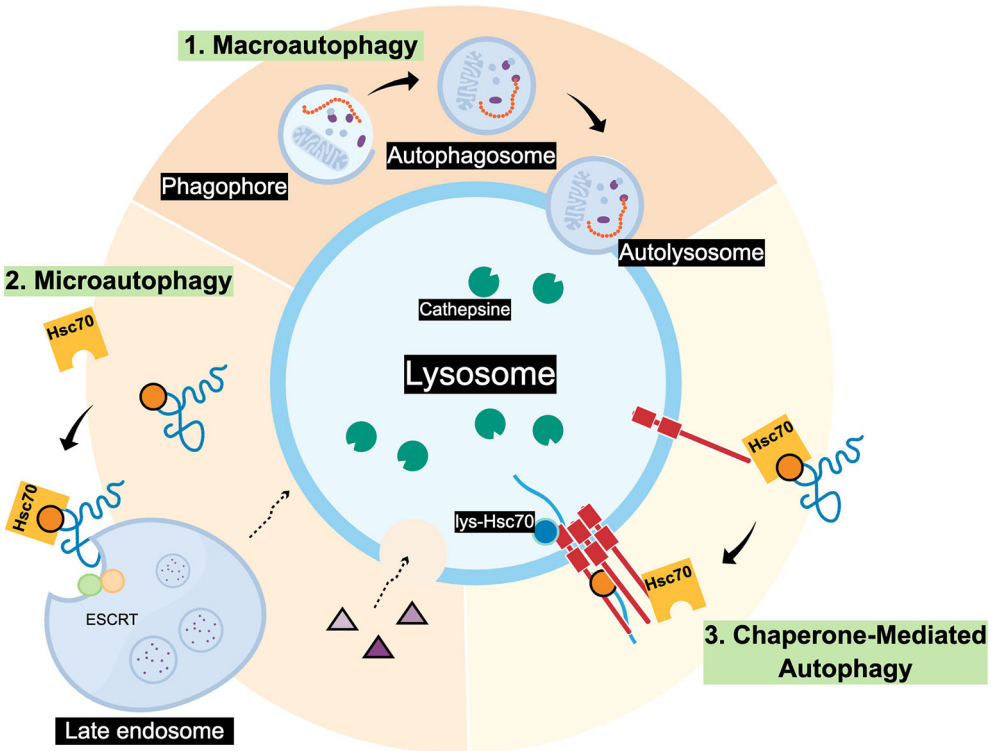


FIGURE 1 | Scheme of three main autophagic pathways. 1. Macroautophagy can degrade individual proteins and complete organelles by engulfing them within an initial membrane called Phagophore, this membrane closes forming the Autophagosome vesicle. Later, the Autophagosome fuses with the lysosome membrane forming the Autolysosome where the cargo will be degraded by cathepsins and other lysosomal proteases; 2. Microautophagy, that in mammals involves the invagination of proteins (blue) that may contain a KFERQ-like motif recognized by Hsc70. Also, the ESCRT complex present in the membrane of late endosomes can form multivesicular bodies (MVBs) containing the cargo to be degraded. Later, MVBs can fuse with the lysosome. In yeast it has been observed that Microautophagy process occurs by direct invagination of the lysosomal membrane to degrade the cargo in the lumen; 3. Chaperone-mediated autophagy (CMA), necessarily requires that substrate proteins possess KFERQ-like motifs to be recognized by Hsc70. Contrary to Microautophagy, CMA also requires the Lamp2A lysosomal protein receptor located at the lysosomal membrane (red) to translocate the unfolded substrate (blue) into the lysosome lumen.

known as phagosomes or autophagosomes that then fuse with the lysosome for the final cargo degradation (2); ii) Microautophagy, the lysosomes directly engulf cargo by membrane invagination (yeast) or late endosomes that form multivesicular bodies (mammals) to capture specific cytosolic components and then fuse with the lysosome for its degradation (3, 4); and (iii) Chaperone-mediated autophagy (CMA), there are no trafficking membranous vesicles involved, instead, cargo is selectively recognized by a chaperone protein and then internalized into the lysosome for its degradation (5). In the case of Microautophagy and CMA, both pathways need the interaction between the KFERQ-like motive in the protein substrate and the Hsc70 for target degradation. However, Microautophagy, does not requires Lamp2A as protein receptor (**Figure 1**) [for a compressive review see (5)].

The evidences reveal that alterations in autophagy are implicated in multiple human diseases and pathophysiological conditions like neurodegeneration (6), infection, immunity and inflammation (7), cardiovascular diseases (8), respiratory affections (9), liver diseases (10) and cancer (11). In the case of CMA, this pathway has been reported to be altered, principally, in neurodegeneration (12), metabolic disorders (12) and cancer (13). In this review, we will focus in the observations that link CMA with cancer. In particular, we will discuss the relevance that changes in CMA substrates and principal components would have in the context of different cancer types.

CHAPERONE MEDIATED AUTOPHAGY (CMA)

The main feature of this particular lysosomal pathway is the cargo selectivity. CMA degrades mostly cytosolic proteins that are characterized by the presence of a consensus pentapeptide known as the KFERQ-like motif. This motif includes at least two positively charged residues (K, R), up to two hydrophobic residues (F), one negatively charged residue (E) and a glutamine (Q) that can be positioned at the beginning or at the end of the motif. Proteins containing the KFERQ-like motif are recognized by the Heat Shock Cognate 70 (Hsc70) protein and other co-chaperones (14) that assist in the transport to the

lysosomal surface to interact with the cytosolic tail of Lysosome-associated membrane protein type 2A (Lamp2A) (5).

Lamp2A is found in a monomeric state at the lysosomal surface and, when the substrate-chaperone-Lamp2A complex is formed, Lamp2A initiates a multimerization process (15). This process is assisted in the cytosolic side by positive regulators including Glial Fibrillary Acidic Protein (GFAP), PH domain Leucine-rich repeat-containing Protein Phosphatase 1 (PHLPP1), and Elongation Factor 1α (EF1α) (16) that regulate and stabilize the multimer. Once stabilized, the Lamp2A multimer serves as a "*translocation complex*" by which the substrate is unfolded and translocated into the lysosomal lumen. Inside the lysosome, the presence of a luminal chaperone lys-Hsc70 is also required to complete the substrate internalization (14, 17) (**Figure 2**).

CMA ACTIVITY IN DIFFERENT CANCER TYPES

Altered CMA activity has been reported in different physiopathological processes such as metabolic alterations, neurodegeneration and aging (12), where the abnormal degradation of the substrates are contributing to the progression of the cellular damage. Changes in CMA activity also has been addressed for different types of cancer. For example, through immunostaining and fluorescent reporter proteins, studies in several cancer cell lines such as lung (A549, H460), breast (MCF7), liver (HUH7), epithelial (Saos 2 osteosarcoma), stomach, colon, uterus, and ovary, have concluded that CMA activity is constitutively upregulated in cancer cells (18). Interestingly, such up-regulation seems to be independent of the macroautophagy status, suggesting a specific role of CMA in these cancer types (19–21). Taking into account the different cancer cell lines studied, it was possible to suggest that CMA is found to be upregulated 2.8-fold with respect to non-oncogenic control cells (18). In this sense, it becomes important to understand the role that CMA up-regulation can be playing in the development of cancer pathology. The most accepted theory is that CMA contributes to maintain the malignant condition by preventing the entry of the cells into an apoptotic process and protecting them from stress (13, 22). Taking into account these two aspects, and the observations made by some authors (18), CMA up-regulation could be considered as a pro-survival cancer cell factor and thus its activity inhibition could be the target for therapeutics. However, the latter is not a general rule since recent studies

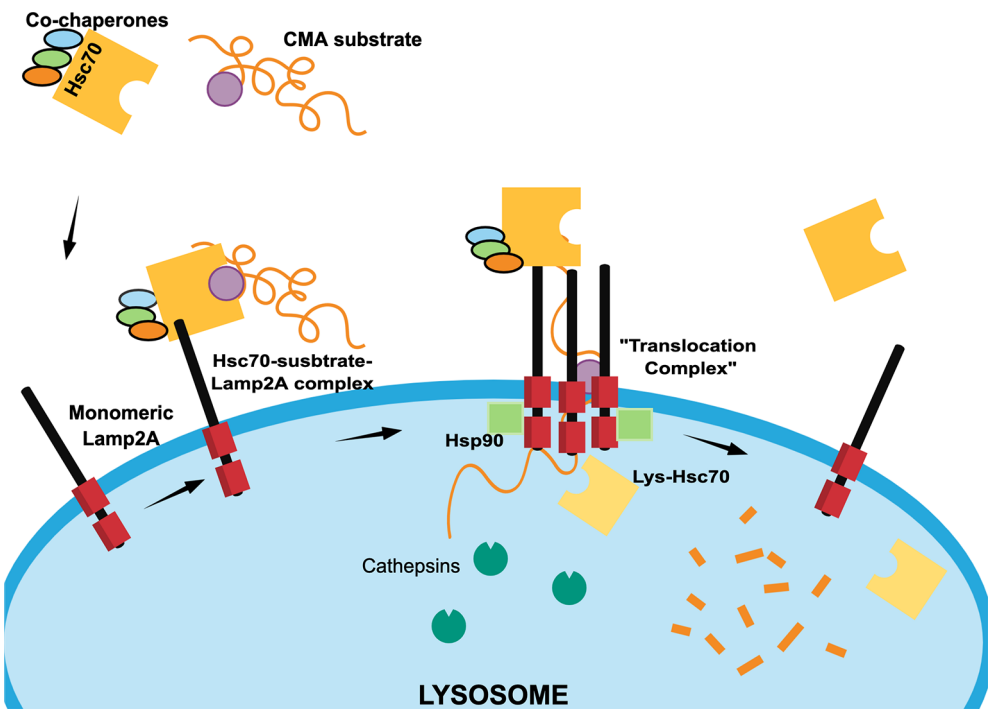


FIGURE 2 | Scheme of different chaperone-mediated autophagy (CMA) steps. In the cytosol, Hsc70 heat shock protein recognizes CMA protein substrates containing KFERQ-like motifs (purple circle) with the assistance of co-chaperones Hip, Hop and Hsp40 (light blue, green and orange ovals) to form a "Hsc70-substrate complex". The Hsc70-substrate complex interacts with monomeric Lamp2A at the lysosomal membrane to induce the formation of a "translocation complex" through Lamp2A oligomerization, which is regulated at the outer lysosome membrane by the Glial Fibrillary Acidic Protein (GFAP), Protein Phosphatase 1 (PHLPP1), and Elongation Factor 1α (EF1α) proteins (not shown). Once the "*translocation complex*" is formed, the CMA substrate protein unfolds to enters at the lysosomal lumen thanks to the assistance luminal Hsc70 (lys-Hsc70) and Hsp90 (green squares). Once, the CMA substrate protein is degraded by cathepsins and other proteases, the "*translocation complex*" dissociates, Hsc70 is recycled and Lamp2A returns to a monomeric state.

also indicate that CMA has an anti-oncogenic role by limiting malignant transformation in some types of cancer (13, 23).

On the other hand, a very recent review describing the role of CMA in regulating the cell cycle by keeping the balance between negative and positive cell cycle regulators suggests that a CMA impairment could lead to tumor progression and cell malignant transformation (24). Further, an additional report has recently showed that CMA is able to drive the balance between stem cell proliferation and cell differentiation, two cellular properties that are closely related with cancer (25).

Thus, up to date, the exact role of CMA in cancer is uncertain and it will depend on each cancer type. Probably, a way to determine how CMA is participating in the progression of cancer is to correctly interpret the significance that alterations of specific CMA substrates or CMA components might have in the different cancer types.

CMA SUBSTRATES IN CANCER: DIFFERENT PERSPECTIVES TO APPROACH THE PROBLEM

Different confirmed or potential CMA substrates in different cancer types accumulate despite the fact that CMA activity is increased (26). Some of these CMA substrates are pro-oncogenic and are protected from CMA degradation by different reasons. Thus, cancer progression not always will depend on the status of CMA activity but also on the altered CMA-dependent degradation of the anti- and/or pro-oncogenic protein substrates (**Table 1**).

One of the most known CMA substrate that is protected from CMA degradation is P53, an important tumor suppressor protein encoded by the *tp53* gene. Whereas wild type P53 protein is a poor CMA substrate, missense mutations in the *tp53* gene leads to a mutant form of P53 which favors its degradation by CMA in the cytosol. Mutant P53 accumulates in the nucleus exerting its pro-oncogenic role and protecting itself from degradation, but under conditions of stress and autophagy inhibition, mutant P53 translocate to the cytosol and interacts with Hsc70 followed by CMA degradation (27). The latter example suggests that, in particular cases, a strategy to counteract the cancer progression can be to expose the mutant pro-oncogenic substrate to be degraded by CMA.

In addition, there is evidence indicating that alteration of CMA substrates through post-translational modifications such as acetylation or phosphorylation can also represent an appropriate alternative to reduce cell malignance. For example, in prostate cancer cells, the up-regulation of HSD17B4, a protein involved in fatty acid oxidation, is correlated with tumor progression (28). HSD17B4 acetylation enhances its interaction with Hsc70 and Lamp2A, allowing its degradation through CMA. However, it was shown that deacetylation processes that are predominant in prostate cancer (28) protects HSD17B4 from CMA degradation and contributes with cancer progression.

TABLE 1 | Protein acting as pro-oncogenic or anti-oncogenic factors, validated or not as chaperone-mediated autophagy (CMA) substrate and its role in different cancer types.

Cancer type	Altered protein in cancer	Normal function	Activity in cancer	KFERQ motif	Protected from degradation	Reference
Breast, Ovarian, cancers Fibrosarcome	**Mutant P53**	Apoptotic pathway	Promoter	Non canonical Proved CMA substrate	Yes	Vakifahmetoglu-norberg et al. (27)
Breast, Prostate cancer	**HSD17B4** 17b- hydroxysteroid dehydrogenase type 4	Fatty acid β-oxidation and steroid metabolism	Promoter	Non canonical Not proved CMA substrate	Yes	Huang et al. (28)
Acute Myeloid leukemia erythroleukemia	**HKII** Hexokinase II	Glucose metabolism	Promoter	Canonical Proved CMA susbtrate	Yes	Xia et al. (29)
Breast cancer	**MORC2** MORC family CW-type zinc finger 2	Lipogenesis and adipogenesis	Promoter	Canonical Proved CMA susbtrate	Yes	Yang et al. (30)
Pancreatic cancer	**IGF-IR** insulin-like growth factor 1 receptor	Proliferation and cell growth	Promoter	Non canonical Not proved	Yes	Xue et al. (31)
Hepatocellular carcinoma Cervical cancer	**HIF 1α** Hypoxia-inducible factor 1α	Hypoxia response Cell cycle regulator	Promoter	Non canonical Proved CMA substrate	No	Hubbi et al. (32)
Gastric cancer	**RND3** Rho Family GTPase 3	Anti-proliferative Cell cycle regulator	Suppressor	Canonical Proved CMA substrate	No	Zhou et al. (33)
Breast cancer	**ATG-5** Autophagy-related gene 5	Autophagy	Suppressor	Non canonical	No	Han et al. (34)
Colorectal	**SNX10/P21** Sorting nexin 10/protein 21	endosome/lysosome homeostasis/ cell cycle regulator	Suppressor	Non canonical Proved CMA substrate	No	Zhang et al. (35)

Phosphorylation is another modification that protects CMA substrates from degradation. Hexokinase II (HKII) is a key enzyme involved in glucose metabolism and is highly expressed in cancer cells (36). Interestingly, in breast cancer, HKII phosphorylation protects the protein from its degradation through CMA, promoting proliferation, migration and tumor growth (37). However, when using a specific HKII phosphorylation inhibitor, tumor growth is reduced (37). In addition, in gastric cancer cells, some members of the Rho family like RND3, are maintained in the cytosol by specific phosphorylation events, which in turn allow their interaction with CMA components followed by lysosomal degradation (38). A similar example is the novel oncogene MORC2 (MORC family CW-type zinc finger 2), that has been found at high levels in breast cancer promoting metastatic progression. This protein is protected from CMA degradation by a phosphorylation that prevents its interaction with Hsc70 (39). Thus, the inhibition of MORC2 phosphorylation to allow its degradation through CMA could be a good approach to reduce the cell malignance.

Finally, to expose the KFERQ-like motif can be also an option to drive the degradation of a pro-oncogenic CMA substrate. For example, it was shown that inhibition of macroautophagy in ovarian cancer cells in the absence of glucose exposes the HKII KFERQ-motif and allows its recognition by Hsc70, directing its degradation through CMA (29). A similar situation occurs with the IGF-IR (insulin-like growth factor-1), a family member of insulin receptors that is enhanced in different cancer types (39). In pancreatic cancer, IGF-IR acts as pro-oncogene and remains protected from CMA degradation by its interaction with chaperone Hsp90. If this interaction is disrupted by specific Hsp90 inhibitors, IGF-IR is exposed for Hsc70 recognition and is degraded through CMA (31).

There are cases where the direct CMA up-regulation can effectively drive the degradation of a particular CMA substrate to prevent cancer progression. Hypoxia-inducible factor 1 (HIF-1), is a transcription factor composed by two subunits, HIF-1α and HIF-1β and is required as an adaptive response to low oxygen conditions and necessary for tumor progression (40, 41). Hsc70 and Lamp2A knock-down led to an increase of HIF-1α protein level in cancer cell lines such as HeLa and Hep3b (32). On the other hand, high expression levels of Hsc70 and Lamp2A in the same cell lines were associated with reduced protein of HIF1α (32). Thus, in this case, the direct enhancement of CMA activity can promote the degradation of this pro-oncogenic protein. Another example is the case of RND3 (Rho Family GTPase 3), an anti-proliferative protein that has been previously confirmed as CMA substrate (33). High expression of Lamp2A was correlated with reduced RND3 protein levels in cancer gastric cells, and the silencing of Lamp2A was associated with increased RND3 protein levels and inhibited cell proliferation (33). Therefore, in this particular case, inhibition of CMA activity could be considered as a therapeutic alternative to implement. Autophagy-related gene 5 (ATG5) acts as a tumor suppressor protein and its absence causes tumor generation in different tissues (42), including breast. Also, in breast cancer, Lamp2A protein is abnormally upregulated (43) and plays an important role in cell survival and metastasis (34). Interestingly, in a breast cancer model it was shown that Lamp2A down-regulation induces ATG5 protein levels increase and inhibited cancer cell growth and metastasis (34), suggesting that an inhibition of CMA activity could be an option to reverse the malignant phenotype dependent on ATG5 protein alterations.

Finally, in colorectal cancer cells, Lamp2A expression levels are upregulated as a consequence of the lower protein levels of sorting nexin 10 (SNX10) (35). The latter leads to increased CMA activity and exacerbated degradation of the tumor suppressor protein P21, allowing the proliferation of colon cancer cells (35). Thus, in this particular case, overexpression of SNX10 protein results to be a good alternative to reduce CMA activity and allows the accumulation an anti-oncogenic action of P21.

LAMP2A AND HSC70 IN CANCER BEYOND CMA

As mentioned above, Lamp2A and Hsc70 are two proteins whose role is essential for the CMA pathway, in particular Lamp2A that has been reported to be the limiting step of the pathway (44). Up to date, many evidences have stablished that in addition to evaluate the levels of Lamp2A and Hsc70, additional requirements and functional assays are necessary to determine whether changes in CMA activity occurs in a determined cellular state. In particular, immunoblots of Lamp2A and Hsc70 in total cell lysates or tissue homogenates are less valuable to determine CMA activity as their changes should be confirmed principally in lysosomes (17, 44, 45). Sometimes, in various cell types, a useful indirect indication of CMA activation can be to evaluate the distribution of hsc70-positive lysosomes toward the perinuclear region (46). Additionally, in total cell lysates or tissue homogenates, and particularly when using isolated lysosomes from cells or tissues, changes in the levels of CMA substrates is a good indication of alteration in CMA activity (46). Finally, applying some functional assays such as the use of photoconvertible CMA reporters, the assessment of intracellular protein degradation or the *in vitro* analysis of CMA using isolated lysosomes, are all very well accepted approaches to confirm changes in CMA activity (44).

Thus, considering all the exposed above, additional considerations should be taken before to conclude that alterations in Lamp2A and Hsc70 levels are directly correlated with changes in CMA activity, and the altered expression of these proteins might have a different significance for the cancerous cell. In fact, these proteins would fulfil additional functions in the cell, in particular Hsc70 (47, 48). Next, we discuss about some evidences reporting alterations in Lamp2A and Hsc70 expression but where changes in CMA activity was not clearly confirmed, suggesting that the alteration in Lamp2A or Hsc70 may have a different role in the cancerous cells.

Lamp2A Protein

By alternative splicing, the *lamp2* gene can generate three different isoform proteins; Lamp2A, Lamp2B, and Lamp2C,

where only Lamp2A has been exclusively linked to CMA activity (45). In patients with breast cancer, Lamp2A overexpression was detected in all samples containing cancerous tissues and was correlated to cancer cell survival (43). In addition, Lamp2A has also been used as a marker to measure the lysosomal content in breast carcinomas and was correlated with increased activity of the transcription factor EB (TFEB). Interestingly, in another study in breast cancer showed that 30% of the cancerous samples displayed Lamp2A overexpression (49). Although the exact role of Lamp2A in breast cancer progression should be further clarified, it is clear that this protein is relevant for cancerous cell survival demonstrated by the inhibition of cell migration and invasion in silenced cells (34). In addition, when Lamp-2A was experimentally up-regulated an increase in cell growth was observed (34). In addition to breast cancer, other cancer types have reported altered Lamp2A expression without an evident analysis on the changes in CMA activity. For example, reduced Lamp2A expression was required to avoid apoptosis and promote cell proliferation in hepatocellular carcinoma xenograft growth (50). Further, in several gastric cancer cells, Lamp2A overexpression was proposed as a good marker for early cancer prediction in precancerous lesions (33). Additionally, in Non-Small-Cells Lung Cancer (NSCLC), an increase in Lamp2A expression was observed compared to healthy tissues (21). Further, in Tumor-associated macrophages (TAM), it was demonstrated that supernatants from tumor cells, which contains growth and inflammatory factors, induce Lamp2A up-regulation in TAM and allow its activation, favoring the tumor growth maintenance (51). Lamp2A silencing by shRNA or CRISPR-Cas9 was correlated with an increase in PRDX1 (peroxiredoxin 1) and CRTC1 (CREB-regulated transcription coactivator 1), two factors necessary to promote macrophage pro-tumorigenic activation. Although CMA activity was not evaluated, the increase in PRDX1 and CRTC1 after Lamp2A down-regulation suggest that in this case a CMA impairment in TAM can be related with tumor growth (51). Further, Lamp2A down-regulation reverted the tumorigenic features of TAM cells (51). Finally, an increased expression of Lamp2A has been found in tissues from patients with colorectal cancer Stage II and III, and the Lamp2A silencing in the colorectal cell line CT26 induced apoptosis and inhibited cell proliferation (19). Taken all together, Lamp2A overexpression is mostly associated to cancer progression independent of observable or reported changes in CMA activity.

Hsc70 Protein

Hsc70 is a cytoplasmic protein that belongs to the Hsp70 multigene family. An important aspect of Hsc70 is that its expression is constitutive and independent to a heat shock response (52). This chaperone has been described to interact with several cancer-related proteins (47) and thus its role in cancer includes its interaction with related and non-related CMA proteins, and with its altered expression levels in cancerous cells or tissue (**Figure 3**). Hsc70 is part of the co-chaperones required for the delivery of "client proteins" to Hsp90 machinery (53). Further, the development of Hsp90 inhibitors has been the focus of many studies due to the implications of this protein in cancer progression (54). However, the inhibition of Hsp90 might induce Hsc70 overexpression which counteract the antitumorigenic effect of the Hsp90 inhibitor (55). Thus, the silencing of Hsc70 in different cancer cell lines, together with the use of Hsp90 inhibitors, has been used to cause cell cycle arrest and increase in tumoral cell apoptosis in response to Hsp90 inhibitors (55). In line with this, it was observed that the use of a Hsp90 inhibitor in B-lineage acute lymphoblastic leukemia cells allowed the induction of Hsc70, cathepsin D inhibition and apoptosis induction of the cancerous cells (56). In addition, BAG-1 a multifunctional protein related to diverse cancer types (57), also interacts with the Hsc70 amino-terminal ATP-binding domain (58). When using peptides resembling the BAG domain in breast cancer cells, the interaction between BAG-1 and Hsc70 is prevented and cell growth is arrested (59). Moreover, the interaction of Hsc70 with glioma pathogenesis-related protein (GLIPR1) was associated with oncogenic signaling pathway by suppressing the transcription of AURKA and TPX2 by SP1 and c-Myb destabilization (60). Furthermore, it has been observed that Rab1A overexpression is vital for the maintenance of cancer cell physiology under stress conditions. With this regard, the ubiquitination and protein degradation of Rab1A was shown to be protected due to its interaction with Hsc70 (61). Thus, in some cancer types, Hsc70 expression can favor cancer progression by, for example, replacing the function of a related protein (e.g., Hsp90) or by protecting a pro-oncogenic factor from its degradation.

However, in other cancer types, Hsc70 can be beneficial by promoting cancer arrest. For example, the overexpression of GKN2 protein sensitized gastric cancer cells to apoptosis under oxidative stress inducers. However, the effect of GKN2 to induce apoptosis was dependent on its interaction with Hsc70 (62). Also, Hsc70 has also been described as an important player in the mechanism regulating the suppression of colorectal cancer metastasis by its interaction with the amino-terminal enhancer of split protein (Aes) (63). Finally, it was shown that colon cancer tissues displayed increased expression and protein complexes of Hsp70, Hsp90 and HOP compared with control tissues, suggesting an association between cancer progression and complexes formation (64).

Additionally, some works have associated the variations in Hsc70 expression as part of cell-to-cell communication, or as a biomarker of cancer progression. In healthy cells, Hsc70 remains intracellular but in the lymphoblastic cell line K562 and in human breast cancer cells, Hsc70 is secreted to the media and considered as a new signal of growth arrest when cells are confluent (65, 66). Hsc70 has also been proposed as a biomarker for endometrial carcinoma due to its up-regulation in cancer tissues (67). Further, the progression of human brain glioma cells and tumor grade was associated with Hsc70 overexpression. In particular, the migration and invasion of these cells was inhibited by a Hsc70 downregulation (68).

Altogether, these evidences further indicate that, depending on each cancer nature, the altered Hsc70 expression might have

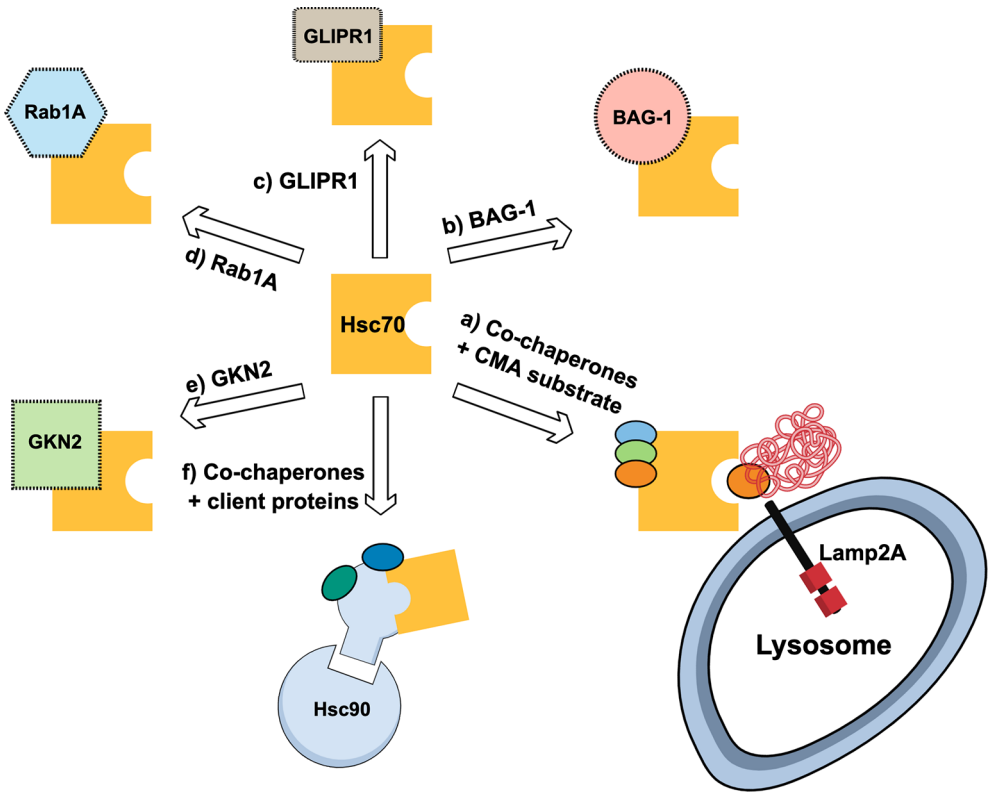


FIGURE 3 | Schematic representation of multiple routes where Hsc70 is associated with cancer. **(A)** shows Hsc70 forming complex with additional chaperones and recognizes chaperone-mediated autophagy (CMA) substrates for lysosomal degradation by Lamp2A-mediated internalization; **(B)** shows Hsc70 interaction with BAG-1, a co-chaperone with anti-apoptotic activity; **(C)** Hsc70 interacts with Glioma Pathogenesis-related Protein 1 (GLIPR1) related to glioma and glioblastoma multiforme; **(D)** Rab1A, a protein related with cancer cell survival, is protected from degradation by interacting with Hsc70, despite Rab1A has canonical KFERQ motif; **(E)** Hsc70 complexes with Gastrokine-2 (GKN2), a secretory protein associated with gastric cancer; **(F)** Hsc70 acting as cochaperone to deliver "client proteins" to Hsc90 machinery.

additional or different effects on cancer progression, rather than modified CMA activity.

CONCLUDING REMARKS

Most of the analyzed evidence, report an up-regulation in Lamp2A and Hsc70 CMA components in the different cancer cell types studied. When the up-regulation of these CMA components were associated with an increase in CMA activity, it was mostly connected with a protective mechanism for stress microenvironment and lack of nutrients, contributing to tumor growth and cancer progression. The latter suggests that inhibition of CMA activity could be a target for therapeutic approaches to increase the sensitivity of tumor cells to stress, promote cell death and decreased tumor growth. However, few evidences also show that CMA activity would contribute to cancer arrest and thus to inhibit or enhance the CMA activity probably will depend on each specific cancer type.

In addition, whereas several proteins involved in the progression of different cancer types have been experimentally well-defined as CMA substrates, others, although having putative KFERQ-like motifs or indirectly associated to changes in CMA activity, have not fully been defined as CMA substrates (**Table 1**). With this regard, although the enhancement or inhibition of CMA can be the objective to reverse the cancer phenotype, also the efforts could be made in modifying protein substrates (e.g., by altering posttranslational modifications, cellular conditions or protein-protein interactions) to promote or inhibit its degradation through CMA, depending on the particular case.

Many evidences have stablished that, in addition to evaluate the levels of Lamp2A and Hsc70, further assays are necessary to determine whether changes in CMA activity occurs in a determined cellular state. According to this, some reports have showed alterations in Lamp2A and Hsc70, without evident assayed changes in CMA activity. The latter leaves the open question about if the changes in Lamp2A or Hsc70 expression in these particular cases can be fulfilling an additional role in carcinogenesis beyond modifying the CMA activity.

In conclusion, depending on each specific cancer type, it will be important to experimentally determine whether detectable

alterations in Lamp2A and Hsc70 expression have a direct impact on CMA activity. If it is the case or not, then the question should be whether targeting CMA activity is an option to counteract cancer progression, or whether the attention of the strategy must be focused on Lamp2A or Hsc70, or some particular CMA protein substrate.

AUTHOR CONTRIBUTIONS

JR and AS analyzed the bibliography, wrote the manuscript, and made figures and table. AA made revisions and editing. MB analyzed the bibliography, contributed with writing, and made substantial revisions and editing of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by CONICYT PIA ACT172066 to MB.

ACKNOWLEDGMENTS

We would like to thank to CONICYT PIA ACT172066 for supporting our research.

REFERENCES

1. Galluzzi L, Baehrecke EH, Ballabio A, Boya P, Bravo-San Pedro JM, Cecconi F, et al. Molecular definitions of autophagy and related processes. *EMBO J* (2017) 36:1811–36. doi: 10.15252/embj.201796697
2. Bento CF, Renna M, Ghislat G, Puri C, Ashkenazi A, Vicinanza M, et al. Mammalian Autophagy: How Does It Work? *Annu Rev Biochem* (2016) 85:685–713. doi: 10.1146/annurev-biochem-060815-014556
3. Mijaljica D, Prescott M, Devenish RJ. Microautophagy in mammalian cells: Revisiting a 40-year-old conundrum. *Autophagy* (2011) 7:673–82. doi: 10.4161/auto.7.7.14733
4. Oku M, Sakai Y. Three Distinct Types of Microautophagy Based on Membrane Dynamics and Molecular Machineries. *BioEssays* (2018) 40:1–6. doi: 10.1002/bies.201800008
5. Tekirdag K, Cuervo AM. Chaperone-mediated autophagy and endosomal microautophagy: Joint by a chaperone. *J Biol Chem* (2018) 293:5414–24. doi: 10.1074/jbc.R117.818237
6. Menzies FM, Fleming A, Caricasole A, Bento CF, Andrews SP, Ashkenazi A, et al. Autophagy and Neurodegeneration: Pathogenic Mechanisms and Therapeutic Opportunities. *Neuron* (2017) 93:1015–34. doi: 10.1016/j.neuron.2017.01.022
7. Levine B, Kroemer G. Biological Functions of Autophagy Genes: A Disease Perspective. *Cell* (2019) 176:11–42. doi: 10.1016/j.cell.2018.09.048
8. Bravo-San Pedro JM, Kroemer G, Galluzzi L. Autophagy and Mitophagy in Cardiovascular Disease. *Circ Res* (2017) 120:1812–24. doi: 10.1161/CIRCRESAHA.117.311082
9. Racanelli AC, Kikkers SA, Choi AMK, Cloonan SM. Autophagy and inflammation in chronic respiratory disease. *Autophagy* (2018) 14:221–32. doi: 10.1080/15548627.2017.1389823
10. Ueno T, Komatsu M. Autophagy in the liver: Functions in health and disease. *Nat Rev Gastroenterol Hepatol* (2017) 14:170–84. doi: 10.1038/nrgastro.2016.185
11. Onorati AV, Dyczynski M, Ojha R, Amaravadi RK. Targeting autophagy in cancer. *Cancer* (2018) 124:3307–18. doi: 10.1002/cncr.31335
12. Alfaro IE, Albornoz A, Molina A, Moreno J, Cordero K, Criollo A, et al. Chaperone mediated autophagy in the crosstalk of neurodegenerative diseases and metabolic disorders. *Front Endocrinol (Lausanne)* (2019) 10:778. doi: 10.3389/fendo.2018.00778
13. Arias E, Cuervo AM. Pros and Cons of Chaperone-Mediated Autophagy in Cancer Biology. *Trends Endocrinol Metab* (2020) 31:53–66. doi: 10.1016/j.tem.2019.09.007
14. Agarraberes FA, Dice JF. A molecular chaperone complex at the lysosomal membrane is required for protein translocation. *J Cell Sci* (2001) 114:2491–9.
15. Bandyopadhyay U, Kaushik S, Varticovski L, Cuervo AM. The Chaperone-Mediated Autophagy Receptor Organizes in Dynamic Protein Complexes at the Lysosomal Membrane. *Mol Cell Biol* (2008) 28:5747–63. doi: 10.1128/mcb.02070-07
16. Juste YR, Cuervo AM. Analysis of chaperone-mediated Autophagy. *Methods Mol Biol* (2019) 1880:621–42. doi: 10.1007/978-1-4939-8873-0
17. Agarraberes FA, Terlecky SR, Dice JF. An intralysosomal hsp70 is required for a selective pathway of lysosomal protein degradation. *J Cell Biol* (1997) 137:825–34. doi: 10.1083/jcb.137.4.825
18. Kon M, Kiffin R, Koga H, Chapochnick J, Macian F, Varticovski L, et al. Chaperone-mediated autophagy is required for tumor growth. *Sci Transl Med* (2011) 3:1–30. doi: 10.1126/scitranslmed.3003182
19. Peng JQ, Han SM, Chen ZH, Yang J, Pei YQ, Bao C, et al. Chaperone-mediated autophagy regulates apoptosis and the proliferation of colon carcinoma cells. *Biochem Biophys Res Commun* (2020) 522:348–54. doi: 10.1016/j.bbrc.2019.11.081
20. Robert G, Jacquel A, Auberger P. Chaperone-Mediated Autophagy and Its Emerging Role in Hematological Malignancies. *Cells* (2019) 8:1–20. doi: 10.3390/cells8101260
21. Suzuki J, Nakajima W, Suzuki H, Asano Y, Tanaka N. Chaperone-mediated autophagy promotes lung cancer cell survival through selective stabilization of the pro-survival protein, MCL1. *Biochem Biophys Res Commun* (2017) 482:1334–40. doi: 10.1016/j.bbrc.2016.12.037
22. Hou T, Fan Y, Dan W, Liu B, Wang Z, Zeng J, et al. Chaperone-mediated autophagy in cancer: Advances from bench to bedside. *Histol Histopathol* (2020) 35:637–44. doi: 10.14670/HH-18-202
23. Tang Y, Wang XW, Liu ZH, Sun YM, Tang YX, Zhou DH. Chaperone-mediated autophagy substrate proteins in cancer. *Oncotarget* (2017) 8:51970–85. doi: 10.18632/oncotarget.17583
24. Andrade-Tomaz M, de Souza I, Rocha CRR, Gomes LR. The Role of Chaperone-Mediated Autophagy in Cell Cycle Control and Its Implications in Cancer. *Cells* (2020) 9:2140. doi: 10.3390/cells9092140
25. Xu Y, Zhang Y, García-Cañaveras JC, Guo L, Kan M, Yu S, et al. Chaperone-mediated autophagy regulates the pluripotency of embryonic stem cells. *Sci (80-)* (2020) 369:397–403. doi: 10.1126/science.abb4467
26. Hao Y, Kacal M, Ouchida AT, Zhang B, Norberg E, Vakifahmetoglu-Norberg H. Targetome analysis of chaperone-mediated autophagy in cancer cells. *Autophagy* (2019) 15:1558–71. doi: 10.1080/15548627.2019.1586255
27. Vakifahmetoglu-Norberg H, Kim M, Xia HG, Iwanicki MP, Ofengeim D, Coloff JL, et al. Chaperone-mediated autophagy degrades mutant p53. *Genes Dev* (2013) 27:1718–30. doi: 10.1101/gad.220897.113
28. Huang H, Liu R, Huang Y, Feng Y, Fu Y, Chen L, et al. Acetylation-mediated degradation of HSD17B4 regulates the progression of prostate cancer. *Aging (Albany NY)* (2020) 12:14699–717. doi: 10.18632/aging.103530
29. Xia HG, Najafov A, Geng J, Galan-Acosta L, Han X, Guo Y, et al. Degradation of HK2 by chaperone-mediated autophagy promotes metabolic catastrophe and cell death. *J Cell Biol* (2015) 210:705–16. doi: 10.1083/jcb.201503044
30. Yang F, Xie HY, Yang LF, Zhang L, Zhang FL, Liu HY, et al. Stabilization of MORC2 by estrogen and antiestrogens through GPER1- PRKACA-CMA pathway contributes to estrogen-induced proliferation and endocrine resistance of breast cancer cells. *Autophagy* (2020) 16:1061–76. doi: 10.1080/15548627.2019.1659609
31. Xue N, Lai F, Du T, Ji M, Liu D, Yan C, et al. Chaperone-mediated autophagy degradation of IGF-1Rβ induced by NVP-AUY922 in pancreatic cancer. *Cell Mol Life Sci* (2019) 76:3433–47. doi: 10.1007/s00018-019-03080-x

32. Hubbi ME, Hu H, Kshitiz, Ahmed I, Levchenko A, Semenza GL. Chaperone-mediated autophagy targets hypoxia-inducible factor-1β(HIF- 1β) for lysosomal degradation. *J Biol Chem* (2013) 288:10703–14. doi: 10.1074/jbc.M112.414771
33. Zhou J, Yang J, Fan X, Hu S, Zhou F, Dong J, et al. Chaperone-mediated autophagy regulates proliferation by targeting RND3 in gastric cancer. *Autophagy* (2016) 12:515–28. doi: 10.1080/15548627.2015.1136770
34. Han Q, Deng Y, Chen S, Chen R, Yang M, Zhang Z, et al. Downregulation of ATG5-dependent macroautophagy by chaperone-mediated autophagy promotes breast cancer cell metastasis. *Sci Rep* (2017) 7:1–11. doi: 10.1038/s41598-017-04994-x
35. Zhang S, Hu B, You Y, Yang Z, Liu L, Tang H, et al. Sorting nexin 10 acts as a tumor suppressor in tumorigenesis and progression of colorectal cancer through regulating chaperone mediated autophagy degradation of p21Cip1/WAF1. *Cancer Lett* (2018) 419:116–27. doi: 10.1016/j.canlet.2018.01.045
36. Patra KC, Wang Q, Bhaskar PT, Miller L, Wang Z, Wheaton W, et al. Hexokinase 2 is required for tumor initiation and maintenance and its systemic deletion is therapeutic in mouse models of cancer. *Cancer Cell* (2013) 24:213–28. doi: 10.1016/j.ccr.2013.06.014
37. Yang T, Ren C, Qiao P, Han X, Wang L, Lv S, et al. PIM2-mediated phosphorylation of hexokinase 2 is critical for tumor growth and paclitaxel resistance in breast cancer. *Oncogene* (2018) 37:5997–6009. doi: 10.1038/s41388-018-0386-x
38. Riento K, Villalonga P, Garg R, Ridley A. Function and regulation of RhoE. *Biochem Soc Trans* (2005) 33:649–51. doi: 10.1042/BST0330649
39. Bergmann U, Funatomi H, Yokoyama M, Korc M, Beger HG. Insulin-like Growth Factor I Overexpression in Human Pancreatic Cancer: Evidence for Autocrine and Paracrine Roles. *Cancer Res* (1995) 55:2007–11.
40. Dewhirst M, Cao Y, Moeller B. Cycling hypoxia and free radicals regulate angiogenesis and radiotherapy response. *Nat Rev Cancer* (2008) 8:425–37. doi: 10.1038/nrc2397.Cycling
41. Semenza GL. HIF-1: upstream and downstream of cancer metabolism. *Curr Opin Genet Dev* (2010) 20:51–6. doi: 10.1016/j.gde.2009.10.009
42. Takamura A, Komatsu M, Hara T, Sakamoto A, Kishi C, Waguri S, et al. Autophagy-deficient mice develop multiple liver tumors. *Genes Dev* (2011) 25:795–800. doi: 10.1101/gad.2016211
43. Saha T. LAMP2A overexpression in breast tumors promotes cancer cell survival via chaperone-mediated autophagy. *Autophagy* (2012) 8:1643–56. doi: 10.4161/auto.21654
44. Arias E. *Methods to Study Chaperone-Mediated Autophagy. 1st ed.* : Methods in Enzymology (2017) 588:283–305. doi: 10.1016/bs.mie.2016.10.009
45. Cuervo AM, Dice JF. Unique properties of lamp2a compared to other lamp2 isoforms. *J Cell Sci* (2000) 113:4441–50.
46. Kaushik S, Cuervo AM. Methods to Monitor Chaperone-Mediated Autophagy. *Methods Enzymol* (2009) 452:297–324. doi: 10.1016/S0076-6879(08)03619-7
47. Liu T, Daniels CK, Cao S. Comprehensive review on the HSC70 functions, interactions with related molecules and involvement in clinical diseases and therapeutic potential. *Pharmacol Ther* (2012) 136:354–74. doi: 10.1016/j.pharmthera.2012.08.014
48. Alessandrini F, Pezzè L, Ciribilli Y. LAMPs: Shedding light on cancer biology. *Semin Oncol* (2017) 44:239–53. doi: 10.1053/j.seminoncol.2017.10.013
49. Giatromanolaki A, Sivridis E, Kalamida D, Koukourakis MI. Transcription Factor EB Expression in Early Breast Cancer Relates to Lysosomal/Autophagosomal Markers and Prognosis. *Clin Breast Cancer* (2017) 17:e119–25. doi: 10.1016/j.clbc.2016.11.006
50. Ding ZB, Fu XT, Shi YH, Zhou J, Peng YF, Liu WR, et al. Lamp2a is required for tumor growth and promotes tumor recurrence of hepatocellular carcinoma. *Int J Oncol* (2016) 49:2367–76. doi: 10.3892/ijo.2016.3754
51. Wang R, Liu Y, Liu L, Chen M, Wang X, Yang J, et al. Tumor cells induce LAMP2a expression in tumor-associated macrophage for cancer progression. *EBioMedicine* (2019) 40:118–34. doi: 10.1016/j.ebiom.2019.01.045
52. Dwornczak B, Mirault ME. Structure and expression of a human gene coding for a 71 kd heat shock "cognate" protein. *Nucleic Acids Res* (1987) 15:5181–97. doi: 10.1093/nar/15.13.5181
53. Wegele H, Müller L, Buchner J. Hsp70 and Hsp90–a relay team for protein folding. *Rev Physiol Biochem Pharmacol* (2004) 151:1–44. doi: 10.1007/s10254-003-0021-1
54. Chaudhury S, Welch TR, Blagg BSJ. Hsp90 as a target for drug development. *ChemMedChem* (2006) 1:1331–40. doi: 10.1002/cmdc.200600112
55. Powers MV, Clarke PA, Workman P. Dual Targeting of HSC70 and HSP72 Inhibits HSP90 Function and Induces Tumor-Specific Apoptosis. *Cancer Cell* (2008) 14:250–62. doi: 10.1016/j.ccr.2008.08.002
56. Xu G, Ma X, Chen F, Wu D, Miao J, Fan Y. 17-DMAG disrupted the autophagy flux leading to the apoptosis of acute lymphoblastic leukemia cells by inducing heat shock cognate protein 70. *Life Sci* (2020) 249. doi: 10.1016/j.lfs.2020.117532
57. Kizilboga T, Baskale EA, Yildiz J, Akcay IM, Zemheri E, Can ND, et al. Bag-1 stimulates Bad phosphorylation through activation of Akt and Raf kinases to mediate cell survival in breast cancer. *BMC Cancer* (2019) 19:1–13. doi: 10.1186/s12885-019-6477-4
58. Sharp A, Crabb SJ, Townsend PA, Cutress RI, Brimmell M, Wang XH, et al. BAG-1 in carcinogenesis. *Expert Rev Mol Med* (2004) 6:1–15. doi: 10.1017/S1462399404007537
59. Sharp A, Cutress RI, Johnson PWM, Packham G, Townsend PA. Short peptides derived from the BAG-1 C-terminus inhibit the interaction between BAG-1 and HSC70 and decrease breast cancer cell growth. *FEBS Lett* (2009) 583:3405–11. doi: 10.1016/j.febslet.2009.09.047
60. Li L, Yang G, Ren C, Tanimoto R, Hirayama T, Wang J, et al. Glioma pathogenesis-related protein 1 induces prostate cancer cell death through Hsc70-mediated suppression of AURKA and TPX2. *Mol Oncol* (2013) 7:484–96. doi: 10.1016/j.molonc.2012.12.005
61. Tanaka M, Mun S, Harada A, Ohkawa Y, Inagaki A, Sano S, et al. Hsc70 contributes to cancer cell survival by preventing Rab1A degradation under stress conditions. *PloS One* (2014) 9:1–11. doi: 10.1371/journal.pone.0096785
62. Zhang Z, Xue H, Dong Y, Zhang J, Pan Y, Shi L, et al. GKN2 promotes oxidative stress-induced gastric cancer cell apoptosis via the Hsc70 pathway. *J Exp Clin Cancer Res* (2019) 38:1–15. doi: 10.1186/s13046-019-1336-3
63. Itatani Y, Sonoshita M, Kakizaki F, Okawa K, Stifani S, Itoh H, et al. Characterization of Aes nuclear foci in colorectal cancer cells. *J Biochem* (2016) 159:133–40. doi: 10.1093/jb/mvv077
64. Kubota H, Yamamoto S, Itoh E, Abe Y, Nakamura A, Izumi Y, et al. Increased expression of co-chaperone HOP with HSP90 and HSC70 and complex formation in human colonic carcinoma. *Cell Stress Chaperones* (2010) 15:1003–11. doi: 10.1007/s12192-010-0211-0
65. Nirdé P, Derocq D, Maynadier M, Chambon M, Basile I, Gary-Bobo M, et al. Heat shock cognate 70 protein secretion as a new growth arrest signal for cancer cells. *Oncogene* (2010) 29:117–27. doi: 10.1038/onc.2009.311
66. Barreto A, Gonzalez JM, Kabingu E, Asea A, Fiorentino S. Stress-induced release of HSC70 from human tumors. *Cell Immunol* (2003) 222:97–104. doi: 10.1016/S0008-8749(03)00115-1
67. Shan N, Zhou W, Zhang S, Zhang Y. Identification of HSPA8 as a candidate biomarker for endometrial carcinoma by using iTRAQ-based proteomic analysis. *Onco Targets Ther* (2016) 9:2169–79. doi: 10.2147/OTT.S97983
68. Sun G, Cao Y, Xu Y, Huai D, Chen P, Guo J, et al. Overexpression of Hsc70 promotes proliferation, migration, and invasion of human glioma cells. *J Cell Biochem* (2019) 120:10707–14. doi: 10.1002/jcb.28362

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Rios, Sequeida, Albornoz and Budini. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Oncology

MINI REVIEW
published: 19 February 2021
doi: 10.3389/fonc.2020.622956

Autophagy-Mediated Exosomes as Immunomodulators of Natural Killer Cells in Pancreatic Cancer Microenvironment

Daniela L. Papademetrio[1,2]*, *María Noé Garcia*[1,2], *Daniel Grasso*[2,3] *and Élida Alvarez*[1,2]

[1] Departamento de Microbiología, Inmunología, Biotecnología y Genética, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina, [2] CONICET, Instituto de Estudios de la Inmunidad Humoral (IDEHU), Universidad de Buenos Aires, Buenos Aires, Argentina, [3] Departamento de Ciencias Biológicas, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina

Pancreas ductal adenocarcinoma is a highly aggressive cancer with an incredible poor lifespan. Different chemotherapeutic agents' schemes have been tested along the years without significant success. Furthermore, immunotherapy also fails to cope with the disease, even in combination with other standard approaches. Autophagy stands out as a chemoresistance mechanism and is also becoming relevant as responsible for the inefficacy of immunotherapy. In this complex scenario, exosomes have emerged as a new key player in tumor environment. Exosomes act as messengers among tumor cells, including tumor microenvironment immune cells. For instance, tumor-derived exosomes are capable of generating a tolerogenic microenvironment, which in turns conditions the immune system behavior. But also, immune cells-derived exosomes, under non-tolerogenic conditions, induce tumor suppression, although they are able to promote chemoresistance. In that way, NK cells are well known key regulators of carcinogenesis and the inhibition of their function is detrimental for tumor suppression. Additionally, increasing evidence suggests a crosstalk between exosome biogenesis and the autophagy pathway. This mini review has the intention to summarize the available data in the complex relationships between the autophagy pathway and the broad spectrum of exosomes subpopulations in pancreatic cancer, with focus on the NK cells response.

Keywords: pancreatic cancer, autophagy, exosomes, natural killer cells, tumor microenvironment

OPEN ACCESS

Edited by:
Zhengfei Zhu,
Fudan University, China

Reviewed by:
Elena Masselli,
University of Parma, Italy
Alessandro Carrer,
Veneto Institute of Molecular Medicine (VIMM), Italy

***Correspondence:**
Daniela L. Papademetrio
dpapademetrio@ffyb.uba.ar

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *29 October 2020*
Accepted: *30 December 2020*
Published: *19 February 2021*

Citation:
Papademetrio DL, Garcia MN, Grasso D and Alvarez É (2021) Autophagy-Mediated Exosomes as Immunomodulators of Natural Killer Cells in Pancreatic Cancer Microenvironment. Front. Oncol. 10:622956. doi: 10.3389/fonc.2020.622956

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive tumor with a survival of 4-6 months after diagnosis (1, 2). Besides the lack of early diagnosis, the rapid development of chemoresistance makes PDAC one of the highest deadly cancers (3). PDAC cells show high levels of basal autophagy (4–6) and an immunosuppressive tumor microenvironment (TME) conditioned by the presence of immunosuppressive cells, i.e., regulatory T lymphocytes (Tregs), tumor-associated macrophages, and myeloid-derived suppressor cells (MDSCs) widely present in the early stages of the disease (7, 8). Immunotherapy against CTLA4 and PD-L1, with remarkable results on several solid tumors

(9, 10), was unsuccessful in PDAC patients (11), even in combination with other standard approaches (12).

Macroautophagy, hereafter named simply as autophagy, is a catabolic process with the capacity to degrades cellular constituents including whole organelles (13, 14). Briefly, upon autophagy induction the serin kinase activity of ULK1 complex induces a successive recruitment of autophagy proteins to discrete areas of ER membrane. This includes a complex with phosphatidyl-inositol 3-kinase activity (PI3K), composed of Beclin-1, ATG14, Vps150 and Vps34, which in turn deposit phosphatidyl-inositol 3-phosphate (PI3P) which is recognized by further autophagic proteins (13, 14). These events let to evolution of the isolation membrane followed by WIPI1/2-mediated recruitment of ATG5-12-16L complex, needed for the incorporation of the lipidated form of LC3B to the budding membrane. Isolation membrane invaginates over the cargo in a LC3-decorated double membrane vesicle denominated the autophagosome. Eventually, the autophagosome fuses with a lysosome where cargo is degraded (13, 14). Literature is contradictory about the role of autophagy in cancer and then it is accepted to be a context dependent factor (6, 15). *In vitro*, gemcitabine which is the standard chemotherapeutic agent against PDAC increases the autophagic flux in PDAC cells in order to avoid its deleterious effects (4). Then, autophagy inhibition increases the sensitivity of PDAC cells to gemcitabine, but also to other treatment such as inhibitors of the NF-kB and MAPK pathways (5).

Extracellular vesicles (EVs) can be classified by size as small (sEVs - <200 nm) or medium/large (m/lEVs - >200 nm), but they can also be discriminated by density, membrane markers and cell type origin (16). From that plethora of different types of EVs, exosomes are those sEVs with an endosomal origin and a size ranging from 30 to 200 nm (17). The biogenesis of these vesicle is produced by inward budding of the membrane of late endosomes/multivesicular bodies (MVBs) (18), These particles are secreted by most cells, including tumoral cells, and can exert an effector response in distant tissues (19, 20). Exosomes transfer their content specifically to target cells, through mechanisms including ligand/receptor recognition, direct fusion with the recipient cells, phagocytosis, endocytosis (21–24). Moreover, they can transfer receptors from the plasma membrane (25) and deliver, to target cells, proteins (26), mRNAs, miRNAs (27, 28) and reporters genes (29). The mechanism that controls the inclusion of specific molecules within the exosomes remains to be clarified, and in addition, a cell can secrete diverse exosome populations each one with a unique content. The so-called tumor-derived exosomes or TEXs often bear tumor associated antigens and in some cases they can activate and stimulate immune cells (30, 31). However, the effect of TEXs over the immune system is not always activation (32). Hence, TEXs can induce apoptosis of effector T cells (33–37), inhibit the cytotoxic activity of natural killer (NK) cells (38–40), activate immunosuppressive functions in myeloid cells (24, 41, 42), impair differentiation of dendritic cells (43), and induce the response of Tregs (44, 45).

NK cells are a class of innate lymphocytes cells with the ability to rapidly eliminate infected and tumor cells. There are two main subclasses of NK cells, phenotypically and functionally different. Phenotypically they can be classified according to the level of CD56 and CD16 expression and functionally according to their cytotoxic potential (46). The $CD56^{dim}CD16^{bright}$ NK cells subset is highly cytotoxic and expresses high levels of perforin and granzyme B. This subpopulation does not migrate to secondary lymphoid organs, but they express chemokine receptors that allow them to migrate to inflamed tissues, can mediate ADCC processes, and have low cytokine secretion capacity. By contrast, $CD56^{bright}CD16^{dim}$ NK cells are the cytokine secreting subset, do not migrate to inflamed tissues and their cytotoxic capacity is limited (46). Opposite to LT $CD8^{+}$, mature NK cells do not need previous activation to exert their functions. Moreover, NK functionality is independent of the presence of non-self-antigens presented by MHC molecules to CD8+ lymphocytes. Hence, NK cells can eliminate target cells without previous sensitization. Nonetheless, it is now well-known that previous activation enhances NK cell activity by regulating the expression of cytotoxic mediators, as well as several receptors (47). Furthermore, previous exposure to haptens, viral infection (HCMV) or cytokines (IL12, IL15, and IL18) generates adaptive NK cells with immunological memory (47).Nevertheless, the potent immunosuppressive TME in PDAC impairs NK function and cytotoxicity by different ways such as downregulation of effector molecules and activation receptor (48). Altogether, NK cells are serious candidates to develop therapeutic strategies to eliminate tumors that are invisible for T cells.

The last few years have seen little or no progress in the development of more effective treatments for patients with PDAC. In this review, we aim to analyze the complex relationships between autophagy and the broad spectrum of exosomes in TME of PDAC, with focus on NK cell response.

PANCREATIC CANCER CELLS ARE MODULATED BY TME-DERIVED EXOSOMES

There is a complex and dynamic relationship among tumor autophagy, immune response and TME. TME is a complex system that is affected by several factors including hypoxia, acidosis, and immune and inflammatory responses. Moreover, TME influences cell adhesion, invasion, angiogenesis, and even tumor autophagy which in turn can promote tumor growth and enhance metastasis. TME is responsible for release of the chemoattractant factors that recruit the immune effector cells. The response of tumor autophagy to the inflammatory components is unpredictable and the generation of a pro-inflammatory environment may not always be effective against the tumor. For example, IL-1 can inhibit the cyclooxygenase 1 (COX-1) signaling pathway, and phosphorylation of the κB inhibitor (IκB), promoting tumor development and metastasis. In contrast, inhibition of IL-1 expression in tumor cells induces overexpression of p21 and p53, leading to tumor suppression

(49). At the pancreatic level, IL-1β induces autophagy in acinar pancreatic cells (50). In the case of pancreatic tumors, they are in hypoxic TME which induces autophagy. In this setting, tumors were reported to increase their autophagy levels in order to selectively degrade granzyme B released by NK cells, thereby inhibiting one of the cytotoxic mechanisms of NK cells (51, 52).

One important member of TME are the cancer-associated fibroblasts (CAFs) which foster proliferation (53) and chemoresistance (54) in PDAC. CAFs are innately insensitive to gemcitabine and a key player in the development of chemoresistance in tumor cells. Exosomes released by gemcitabine-treated CAFs increase proliferation and survival of PDAC cell lines by carrying the chemoresistance-inducing factors, Snail, and miR-146a, which in turn also induce its own expression in the recipient cells (54). Moreover, CAFs-derived exosomes contain the miR-106b which promote gemcitabine resistance in PDCA cells by targeting TP53INP1 (55).

Recently, the exosomes from bone marrow mesenchymal stem cell (BMSC), residents of the TME, raised attention in PDAC. The over-expression of miR-126-3p in BMSC-derived exosomes not only inhibits the proliferation, invasion and metastasis of PDAC cells, but also promotes apoptosis *in vitro* and *in vivo* by down-regulation of disintegrin and metalloproteinase-9, ADAM9 (56). Furthermore, the amount of miR-1231 in those sEVs was significantly correlated with the TNM stage of PDAC in the clinic. The proliferation, migration, invasion, and adhesion to the matrix of PDAC cells were negatively regulated by BMSC-derived exosomes transfected with miR-1231 oligonucleotides. Then, the exosomes extracted from BMSCs, with high levels of miR-1231, inhibit the proliferation of pancreatic cancer cells and induce cell cycle arrest (57). Finally, similar results can be observed in exosomes from the tumor-associated stroma (TAS) cells, which are enriched in miR-145 and possess tumor suppressive properties by inducing apoptosis of PDAC cells (58).

The studies carried out suggest that the ability of exosomes to induce or suppress the proliferation, invasion, metastasis and/or chemoresistance of pancreatic cancer cells, depend on the cell type where those vesicles come from. Different sources of exosomes have different effects on pancreatic cancer cells activity, or even the opposite, which needs further clarification and in-depth study.

RELATIONSHIP BETWEEN AUTOPHAGY AND EXOSOMES BIOGENESIS

Alternative to the direct fusion with lysosomes (see *Introduction*), autophagosomes can previously fuse with some endocytic compartments such as early and late endosomes, and the MVBs. These merged structures, called amphisomes, eventually fuses with lysosomes where sequestered material is finally degraded (59). Therefore, autophagy induction has been shown to cause recycling of the MVBs, which, instead of fusing with the plasma membrane, enter the autophagic pathway. Furthermore, it was observed in some cell lines a relationship between exosomes release and the induction level of the autophagy pathway (60–63). Consequently, it is not surprising to find evidence of autophagic pathway cross-linking with exosome biogenesis (64). Starvation-induced autophagy reduces the release of exosomes in K562 cells (63). Starvation could increase in the fusion between MVBs and autophagosomes, thus directing the MVBs toward the degradative pathway. Similarly, inhibition of PIKfyve kinase, essential for endolysosomal vesicular trafficking, increases exosome release and reduces the degradative process *via* autophagy, probably due to reduced fusion of lysosomes with MVBs and autophagosomes (65). In line with this, the lysosomal dysfunction, provoked by ammonium chloride or bafilomycin A1, increases sEVs secretion of SH-SY5Y cells (66). Nevertheless, we cannot discard that those results are due to vesicular trafficking interference.

There is data supporting that at least part of the autophagy machinery contributes to the biogenesis of exosomes, in a process where completion of the autophagic process itself seems to be dispensable (67, 68). In non-autophagic functions, ATG5 and ATG16L1 proteins have been associated with the biogenesis of exosomes (67). ATG5 participates in the dissociation of the vacuolar proton pump (V1V0-ATPase) from the MVBs preventing its acidification, and this is believed to allow the fusion with the plasma membrane and consequent exosomes releasing. Accordingly, depletion of ATG5 or ATG16L1 significantly reduces exosome release and attenuates exosomal enrichment in LC3B-II. Moreover, lysosomal or V-ATPase inhibitors rescue the release of exosomes in ATG5 depleted cells further supporting the role of luminal pH to define the fate of MVBs. It is interesting to note that while ATG5 decreases the acidification of the MVBs, it increases the acidification in those LC3 positive intracellular compartments, such as autolysosome, phagosomes associated with LC3 and endosomes, all of them destined for degradation. A proposed model indicates that in MVBs, LC3 can remove ATP6V1E1 from intraluminal vesicles/exosomes and decrease acidification, while it recruits ATP6V1E1 or stabilize V1V0ATPase in the aforementioned degradative vesicles to promote acidification (67). The complex of two other autophagy proteins, ATG12 and ATG3, interacts with ALIX and ESCRT-associated proteins, crucial in exosomes biogenesis (68). Hence, loss of ATG12-ATG3 alters the morphology of MVBs, impedes late endosome trafficking, and reduces exosome biogenesis. Worth note that decreased ALIX expression reduces basal autophagic flux, demonstrating reciprocal regulation between both pathways. Interestingly, the lack of ALIX or the ATG12-ATG3 complex impairment do not affect starvation-induced autophagy, suggesting different regulatory machinery for basal and stress-induced autophagy, as well as the interaction of these pathways with endocytic compartments (68).

Highly desmoplastic and poor vascularized, PDAC stroma imposes a hypoxic condition to most cancer cells into pancreatic tissue. It was described that hypoxia, an autophagy inductor, promotes the release of EVs in several PDAC cell lines. The effect seems to be quite specific since a significant increase of sEVs,

without or minimal release of mEVs and lEVs, is observed. Moreover, changes in size distribution among the sEVs is observed with a shift toward smaller average size under extreme hypoxia (69). Furthermore, the GAIP C-terminal interaction protein (GIPC) acts as a scaffold to control receptor-mediated trafficking (70–72). After receptor internalization, GIPC is transiently associated with the pool of endocytic vesicles that are close to the plasma membrane (73). A regulatory role of GIPC on autophagy, *via* the glucose-dependent metabolic pathway, and on biogenesis and release of exosomes has been described in AsPC-1 and PANC-1 pancreatic tumor cells (74). GIPC depletion in these cell lines generates metabolic stress with autophagy induction and increased exosome release. Lack of GIPC increases LC3-II expression and biogenesis of autophagosomes and at the same time leads to increased secretion of exosomes by the PDAC cells. Mechanistically, the absence of GIPC increases exosomes released by higher expression levels of ALIX, TSG101 and CHMP4B. Noteworthy is that exosomes from GIPC-depleted cells lack the drug resistance associated molecule ABCG2, suggesting that this molecule might be a sEVs cargo (74).

Altogether, several molecules that belong from the autophagy pathway seem to play important roles in exosomes biogenesis. However, we still have a long way to precisely define how deeply the autophagy and exosomes biogenesis are crisscrossed. In addition, the stroma profile, for instance through its hypoxic status, let us glimpse that influences of TME over cancer cells in PDAC could be even far more complex that speculated some time ago.

PDAC-DERIVED EXOSOMES INFLUENCE TUMOR BEHAVIOR

Among the cells with different grades of malignancy that compose the PDAC, highly invasive cell-derived exosomes promote the migration and invasion of weakly invasive cells (75). ZIP4, a zinc transporter, is the most up-regulated exosomal protein and promotes the growth of recipient cells (76). Moreover, exosomes derived from highly invasive cells are rich in miR-125b-5p which promotes migration and invasion and is associated with metastasis in PDAC through MEK2/ERK2 signaling (77). Likewise, the miR-5703 present in exosomes from primary cultures of pancreatic stellate cells is capable of fostering proliferation of PDAC cells by activation of the PI3K/Akt pathway. This has been shown to be due to miR-5703 downregulated CMTM4 where CMTM4 suppresses the PI3K/Akt pathway (78). In a different aspect of tumor biology, TEXs can also transfer chemoresistance by a paracrine action. Gemcitabine, being one of the most commonly used chemotherapeutic agents in pancreatic cancer (79), upregulates miR-155 expression in PDAC cells which is transferred, through exosomes, to the neighboring cells. This microRNA confers chemoresistance to receptor cells by upregulation of SOD2 and CAT, involved in ROS detoxification, and downregulation of DCK, a gene related to gemcitabine metabolism (80). Additionally, miR-155 induces the biogenesis and secretion of exosomes leading to a positive feedback loop of drug resistance (80, 81).

The composition, biogenesis and secretion of exosomes are finely regulated processes, influenced by changes in the TME. In this context, exosome secretion is an efficient adaptive mechanism by which cells modulate intracellular stress situations and modify their microenvironment (82). PDAC cells are usually exposed to hypoxia, which is an important autophagy inductor, as commented above. This situation induces adaptation mechanisms that promote endothelial activation, angiogenesis, proliferation, and cell survival (83). Noteworthy, concomitantly to autophagy flux induction, cells under hypoxic or anoxic stress increase the secretion of exosomes rich in CD63, CD9, and miR-210 in breast cancer cells (84). Furthermore, in this situation, secreted exosomes contain proteins associated with cell migration, degradation of the extracellular matrix, growth signaling molecules, clathrin-mediated endocytosis, and molecules of the endothelial and vascular growth factor signaling pathway (85, 86). Acid conditions are common in the tumor microenvironment. This factor also modulates the release, charge, function, and trafficking of exosomes released by the tumor cell. An acidified microenvironment increases the release of exosomes, but with a different lipid composition. These exosomes are enriched in sphingomyelin and GM3 ganglioside, thus increasing their rigidity and fusion efficiency with the target cell (87). It has been shown that situations such as oxidative and thermal stress can increase the release of immunosuppressive exosomes from leukemia cells and T and B lymphomas (32). Besides, the effects of pancreatic cancer-derived sEVs on T lymphocytes are far from be elucidated. The promotion of Treg expansion and impairment of T lymphocytes cytotoxicity against PDAC cells by pancreatic cancer-derived sEVs was recently described. In these lymphocytes, the up-regulation of FOXP3 and the consequent Treg promotion was mediated by the ATM-AMPK-SIRT1/2/6- FOXO1A/FOXO3A axis, suggesting an induction of autophagy pathway by PDAC TEXs (88).

In the TME, the exosomes from hypoxic PDAC cells are capable of activating the PTEN/PI3K pathway, inducing the shifting of macrophages toward the M2 phenotype. This process is dependent on HIF1a or HIF2a, and accelerates invasion, migration and epithelial-mesenchymal transition (EMT) of PDAC cells (89). Moreover, CD151−/tetraspanin 8 containing exosomes support the EMT of non-metastatic PDAC cells for a motile phenotype (90). Furthermore, exosomes bearing VEGF and TGF-β promote angiogenesis enhancing the invasiveness and the establishment of a metastatic TME. PDAC also releases TEXs bearing c-Met (proto-oncogene mesenchymal-epithelial transition factor) and PD-L1 (programmed cell death 1 ligand 1) (91). The tyrosine kinase receptor c-Met controls key signaling cascades including MAPK, STAT, NF-κB and PI3K/Akt pathways, which overall provide proliferation, migration and an anti-apoptotic status of tumor cells (92). On the other hand, PD-L1 is a ligand of the PD-1 receptor which prevents from excessive immune response and guarantees the tolerance of harmless antigens and self-tissues.

Tumor cells take advantage of this mechanism by expressing PD-L1 in order to evade immune control (93). In this context, TEXs from PDAC bear both c-Met and PD-L1 on their surface enhancing the carcinogenesis. Importantly, detection of c-Met and PD-L1 may have diagnostic or prognostic relevance when are detected jointly with the marker CA 19-9 used in PDAC (91).

Finally, PDAC-derived TEXs composition results from activation of several survival pathways which confers aggressiveness, chemoresistance and even immune evasion to neighboring tumor cells.

NATURAL KILLER CELLS IN PDAC MICROENVIRONMENT

NK cells are central in the immunological fight against tumor and infected cells. Although NK cells are expected to play an important role in the immune surveillance against tumors, suppressive components in the TME dampen their efficacy. Several studies proposed Tregs as the responsible in suppressing tumor-infiltrating NK cells (94, 95). However, TEXs have gained attention as key players for immunosuppression in the TME. The exposure of phosphatidylserine (PS) is perhaps the most representative "eat-me" signal which is recognized by opsonins and other serum proteins for removal of apoptotic bodies by phagocytic cell. Physiologically, the externalized PS functions as a dominant immunosuppressive signal, promoting tolerance and preventing local and systemic activation of immune system. Pathologically, the innate immunosuppressive effect of externalized PS has been commandeered by numerous microorganisms to facilitate infection, and in some cases to establish infection latency. In TME, PS is also profoundly dysregulated and inhibits the development of tumor immunity. The exposure of PS is favored by the hypoxic stress, but also PS is exposed in TEXs where it binds to PS-receptors (e.g., TIM-receptors on immune cells), triggering the immune-suppressive signals (i.e. enhanced TGF-β and IL-10 secretion) and leading to an impaired immune activation (96).

NK cells exert their cytotoxic function directly by contact with tumor cells, but also through the action of their own secreted exosomes. Interestingly, the exosomes released by the NK cells seem to be independent of its activation state. Activated and resting NK cells release almost the same number of exosomes, which contain typical protein markers of NK cells such as FasL and perforin. Moreover, these exosomes exert cytotoxic activity against several tumor cell lines *in vitro* (97). In this sense, NK-derived exosomes can regulate tumor cells suppression by two mechanisms: Fas-FasL interaction between exosomes and tumoral cells membrane, and cytotoxicity triggered by excessive uptake of exosomes in the target cells (98). Recently, the presence of miR-3607-3p in EVs was associated with suppression of pancreatic cancer (99). EVs derived from NK cells, enriched in miR-3607-3p, could suppress PDAC development and malignant transformation. The amount of miR-3607-3p in NK cells and its EVs is higher than in PDAC cells, but this miRNA increases significantly in these last when they are cultured in presence of NK-derived EVs (99). IL-26 is a member of the IL-10 cytokine family with unknown function in human tumors. Compared to healthy tissue IL-26 in highly expressed PDAC, miR-3607-3p directly suppresses its expression in these tumoral cells. In sum, there is a significant negative correlation between the expression levels of miR-3607-3p and IL-26 in pancreatic cancer tissues. However, in gastric cancer cells it was reported that over-expression of IL-26 facilitates proliferation and survival by regulation of STAT1/STAT3 signaling (100). Worth to note, that characteristics of cytokine composition in the surroundings where NK cells are activated determine the fate of those immune cells. For instance, survival of NK cells is promoted in presence of IL-15, an innate cytokine, or IL-2, an adaptive cytokine. However, NK cells activated in presence of IL-2 die by apoptosis after contact with vascular endothelium, a key step for their extravasation (101). Further work will elucidate whether the exosomes present in the TME could be mediators of this phenomenon.

NK cells represent a significant attempt of the immune system to fight against PDAC. Nevertheless, cancerous cells, through the PDAC-derived TEXs, can inhibit the functionality of NK cells. In response, NK cells release exosomes which contain FasL and perforin and seem to exert cytotoxic activity against tumor cells. More evidence is needed to completely understand the role of NK-derived exosomes over the PDAC as a whole and vice versa.

Finally, those data could give us the basis to design strategies where this game of different intratumorally exosome populations are exploited for the well-being of patients.

CONCLUSIONS AND PERSPECTIVES

Indeed, the development of specific immunotherapy protocols based on NK cells to treat cancer has been dampened by the complexity of the mechanisms that regulate NK cell function and elimination of target cells. Luckily, times are changing and, at present, in the era of cancer heterogeneity and immunotherapy, NK cells are emerging as the golden effectors to eliminate non-antigenic tumor cell clones. A perfect duet in the symphony of destruction, LTc and NK cells destroy immune "visible" and "invisible" cancer cells to overcome immunogenic tumor heterogeneity. A better understanding of autophagy and exosome pathways and their interrelationships seems to be key for controling these events, where we could find the way of successfully using the immune system against the deadly PDAC.

Available data suggest that exosomes, EVs in general, are changing the communication paradigm within the TME (**Figure 1**). These tiny vesicles can modulate both the immune and therapeutic responses in complex and difficult-to-treat pathologies such as the PDAC. The scientific community is just beginning to understand the mechanisms that govern the intricate and complex interactions among the different actors into the TME. In this scenario, autophagy seems to play a key role in exosomal biogenesis regulation and probably also in cargo selection. We still have a long way to go but is for sure that a

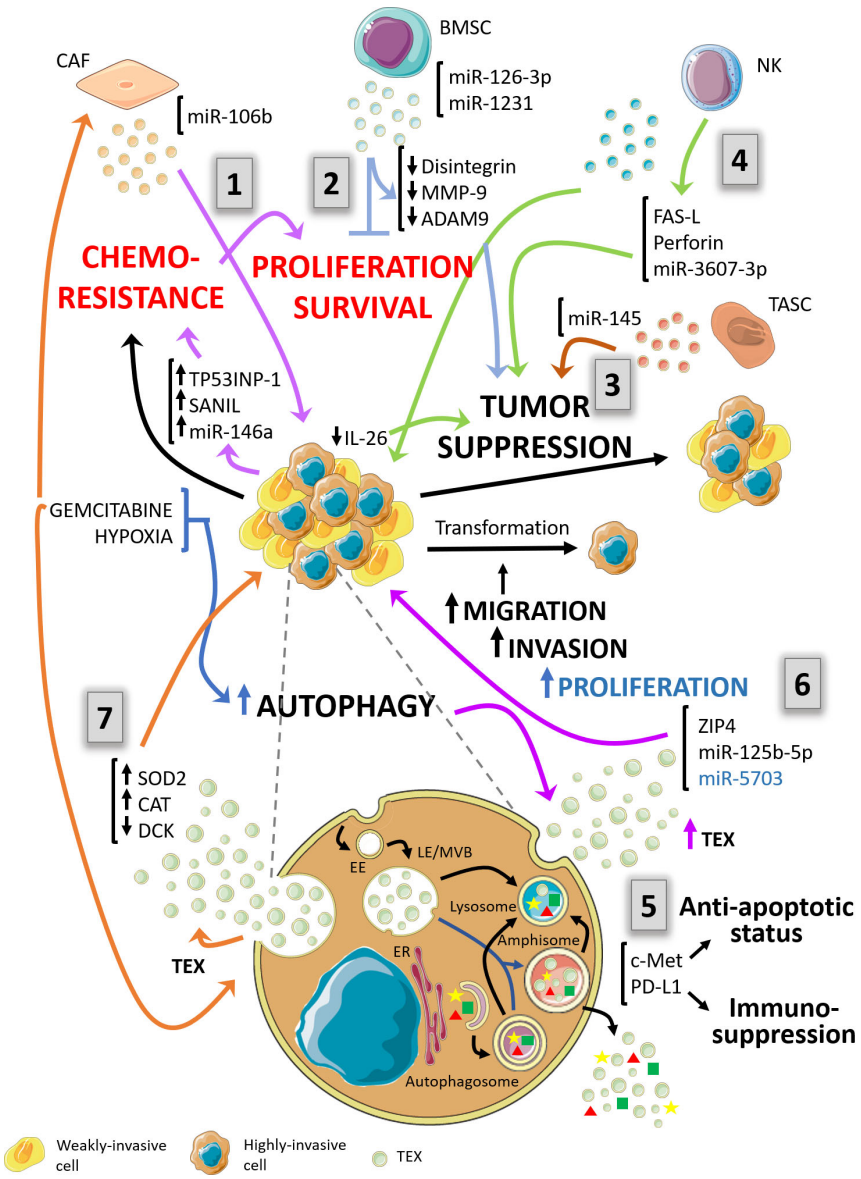


FIGURE 1 | Schematic diagram depicting a proposal model of the complex relationship between autophagy and exosomes in the context of pancreas ductal adenocarcinoma (PDAC) and its environment. The autophagy pathway and exosomes biogenesis are suggested to be highly interconnected. Both pathways share several structures of the endo-lysosomal system. In the context of PDAC and its tumor microenvironment, autophagy and exosomal processes are mutually influenced and condition tumor behavior responding to the pressure of the immune system. (1) – *violet arrows* - Cancer-associated fibroblasts (CAFs) foster proliferation and chemoresistance. Exosomes bearing miR-106b are released by gemcitabine-treated CAFs increasing proliferation and survival of PDAC cell lines. The effector response is traduced in cancer cells as induction of chemoresistance-inducing factors, Snail, and miR-146a, and TP53INP1. (2) – *light blue arrows* - Bone marrow mesenchymal stem cell (BMSC)-derived exosomes contain miR-126-3p and miR-1231, which in turn inhibit proliferation, invasion and metastasis and promotes apoptosis by down-regulation of disintegrin and metalloproteinase-9 (ADAM9). (3) – *brown arrows* - Vesicles from tumor-associated stroma cells (TASC) are enriched in miR-145. This molecule possesses tumor suppressor action on target cells by inducing apoptosis. (4) – *green arrows* - NK-derived exosomes mediate tumor cells suppression by two mechanisms. One of them is Fas-FasL interaction between exosomes and tumoral cells. The other mechanism is mediated by excessive uptake by cancer cells of exosomes carrying miR-3607-3p which possess tumor suppressive qualities and decrease IL-26 expression. (5) – *purple arrows* – PDAC tumor exosomes (TEXs) bearing c-Met and PD-L1 enhance carcinogenesis. c-Met provides proliferation, migration, and an anti-apoptotic status in recipient cancer cells. PD-L1 guarantees evasion of immune control. (6) – *black arrow* – TEXs from highly invasive cells carry ZIP4, miR-125b-5p and miR-5703 towards weakly invasive cancer cells enhancing the aggressiveness of these last and promoting an increased invasive potential. (7) – *orange arrows* – In response to gemcitabine treatment, chemoresistant PDAC cancer cells are capable of transferring their resistance properties to neighboring cells through exosomes. They release TEXs bearing miR-155 and induce upregulation of SOD2 and CAT meanwhile DCK, a gene related to gemcitabine metabolism, is downregulated. Furthermore, exosomes from different cell types of tumor microenvironment condition autophagy response and affect PDAC behavior. EE, Early endosomes; LE/MVB, Late Endosomes/Multivesicular bodies; ER, endoplasmic reticulum; TEX, Tumor-derived exosomes.

future with an exciting new comprehension about tumor biology is waiting.

AUTHOR CONTRIBUTIONS

This review was drafted by DP, MG, DG, and EA, designed by DG and critically revised by DG and EA. Figure was elaborated by DP and MG. All authors contributed to the article and approved the submitted version.

FUNDING

University of Buenos Aires 20020170100014BA.

ACKNOWLEDGMENTS

The authors thank the University of Buenos Aires and the National Council for Scientific and Technological Research (CONICET) for the facilities to carry out our work every day.

REFERENCES

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-tieulent J, Jemal A. Global Cancer Statistics, 2012. *CA Cancer J Clin* (2015) 65:87–108. doi: 10.3322/caac.21262
2. Verslype C, Van Cutsem E, Dicato M, Cascinu S, Cunningham D, Diaz-Rubio E, et al. The management of pancreatic cancer. Current expert opinion and recommendations derived from the 8th World Congress on Gastrointestinal Cancer, Barcelona, 2006. *Ann Oncol* (2007) 18:1–10. doi: 10.1093/annonc/mdm210
3. Ying J, Zhu L, Liu B. Developments in metastatic pancreatic cancer : Is gemcitabine still the standard? *World J Gastroenterol* (2012) 18:736–45. doi: 10.3748/wjg.v18.i8.736
4. Papademetrio DL, Cavaliere V, Simunovich T, Costantino S, Campos MD, Lombardo T, et al. Interplay between autophagy and apoptosis in pancreatic tumors in response to gemcitabine. *Target Oncol* (2014) 9:123–34. doi: 10.1007/s11523-013-0278-5
5. Papademetrio DL, Lompardía SL, Simunovich T, Costantino S, Mihalez CY, Cavaliere V, et al. Inhibition of Survival Pathways MAPK and NF-kB Triggers Apoptosis in Pancreatic Ductal Adenocarcinoma Cells via Suppression of Autophagy. *Target Oncol* (2016) 11:183–95. doi: 10.1007/s11523-015-0388-3
6. Yang S, Wang X, Contino G, Liesa M, Sahin E, Ying H, et al. Pancreatic cancers require autophagy for tumor growth. *Genes Dev* (2011) 25:717–29. doi: 10.1101/gad.2016111
7. Inman KS, Francis AA, Murray NR. Complex role for the immune system in initiation and progression of pancreatic cancer. *World J Gastroenterol* (2014) 20:11160–81. doi: 10.3748/wjg.v20.i32.11160
8. Clark CE, Hingorani SR, Mick R, Combs C, Tuveson DA, Vonderheide RH. Dynamics of the immune reaction to pancreatic cancer from inception to invasion. *Cancer Res* (2007) 67:9518–27. doi: 10.1158/0008-5472.CAN-07-0175
9. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: A common denominator approach to cancer therapy. *Cancer Cell* (2015) 27:450–61. doi: 10.1016/j.ccell.2015.03.001
10. Alsaab HO, Sau S, Alzhrani R, Tatiparti K, Bhise K, Kashaw SK, et al. PD-1 and PD-L1 checkpoint signaling inhibition for cancer immunotherapy: mechanism, combinations, and clinical outcome. *Front Pharmacol* (2017) 8:561. doi: 10.3389/fphar.2017.00561
11. Jindal V. Immune checkpoint inhibitors in gastrointestinal malignancies. *J Gastrointest Oncol* (2018) 9:390–403. doi: 10.21037/jgo.2018.01.03
12. Guo S, Contratto M, Miller G, Leichman L, Wu J. Immunotherapy in pancreatic cancer: Unleash its potential through novel combinations. *World J Clin Oncol* (2017) 8:230–40. doi: 10.5306/wjco.v8.i3.230
13. Bento CF, Renna M, Ghislat G, Puri C, Ashkenazi A, Vicinanza M, et al. Mammalian Autophagy: How Does It Work? *Annu Rev Biochem* (2016) 85:685–713. doi: 10.1146/annurev-biochem-060815-014556
14. Grasso D, Renna FJ, Vaccaro MI. Initial Steps in Mammalian Autophagosome Biogenesis. *Front Cell Dev Biol* (2018) 6:1–10. doi: 10.3389/fcell.2018.00146
15. Grasso D, Garcia MN, Iovanna JL. Autophagy in pancreatic cancer. *Int J Cell Biol* (2012) 2012:1–7. doi: 10.1155/2012/760498
16. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* (2018) 8:1–43. doi: 10.1080/20013078.2018.1535750
17. Pegtel DM, Gould SJ. Annual Review of Biochemistry Exosomes. *Annu Rev Biochem* (2019) 88:487–514. doi: 10.1146/annurev-biochem-013118
18. Kowal J, Tkach M, Théry C. Biogenesis and secretion of exosomes. *Curr Opin Cell Biol* (2014) 29:116–25. doi: 10.1016/j.ceb.2014.05.004
19. Théry C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nat Rev Immunol* (2002) 2:569–79. doi: 10.1038/nri855
20. Tkach M, Théry C. Communication by Extracellular Vesicles: Where We Are and Where We Need to Go. *Cell* (2016) 164:1226–32. doi: 10.1016/j.cell.2016.01.043
21. Gasser O, Schifferli JA. Activated polymorphonuclear neutrophils disseminate anti-inflammatory microparticles by ectocytosis. *Blood* (2004) 104:2543–8. doi: 10.1182/blood-2004-01-0361
22. Segura E, Guerin C, Hogg N, Amigorena S, Thery C. CD8+ Dendritic Cells Use LFA-1 to Capture MHC-Peptide Complexes from Exosomes In Vivo. *J Immunol* (2007) 179:1489–96. doi: 10.4049/jimmunol.179.3.1489
23. Nolte-'t Hoen ENM, Buschow SI, Anderton SM, Stoorvogel W, Wauben MHM. Activated T cells recruit exosomes secreted by dendritic cells via LFA-1. *Blood* (2009) 113:1977–81. doi: 10.1182/blood-2008-08-174094
24. Liu Y, Xiang X, Zhuang X, Zhang S, Liu C, Cheng Z, et al. Contribution of MyD88 to the tumor exosome-mediated induction of myeloid derived suppressor cells. *Am J Pathol* (2010) 176:2490–9. doi: 10.2353/ajpath.2010.090777
25. Baj-Krzyworzeka M, Szatanek R, Węglarczyk K, Baran J, Urbanowicz B, Brański P, et al. Tumour-derived microvesicles carry several surface determinants and mRNA of tumour cells and transfer some of these determinants to monocytes. *Cancer Immunol Immunother* (2006) 55:808–18. doi: 10.1007/s00262-005-0075-9
26. Putz U, Howitt J, Doan A, Goh C-P, Low L-H, Silke J, et al. The Tumor Suppressor PTEN Is Exported in Exosomes and Has Phosphatase Activity in Recipient Cells. *Sci Signal* (2012) 5:ra70–0. doi: 10.1126/scisignal.2003084
27. Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan MLGLG, Karlsson JM, et al. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *J Biol Chem* (2015) 119:756–66. doi: 10.1182/blood-2011-02-338004
28. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* (2007) 9:654–9. doi: 10.1038/ncb1596
29. Skog J, Würdinger T, van Rijn S, Meijer DH, Gainche L, Curry WT, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol* (2008) 10:1470–6. doi: 10.1038/ncb1800
30. Cho J, Lee YS, Kim SH, Ko JK, Kim CW. MHC independent anti-tumor immune responses induced by Hsp70-enriched exosomes generate tumor regression in murine models. *Cancer Lett* (2009) 275:256–65. doi: 10.1016/j.canlet.2008.10.021
31. Nilsson J, Skog J, Nordstrand A, Baranov V, Mincheva-Nilsson L, Breakefield XO, et al. Prostate cancer-derived urine exosomes: A novel approach to biomarkers for prostate cancer. *Br J Cancer* (2009) 100:1603–7. doi: 10.1038/sj.bjc.6605058
32. Hedlund M, Nagaeva O, Kargl D, Baranov V, Mincheva-nilsson L. Thermal- and Oxidative Stress Causes Enhanced Release of NKG2D Ligand-Bearing

Immunosuppressive Exosomes in Leukemia / Lymphoma T and B Cells. *PLos One* (2011) 6:1–10. doi: 10.1371/journal.pone.0016899
33. Albanese J, Meterissian S, Kontogiannea M, Dubreuil C, Hand a, Sorba S, et al. Biologically active Fas antigen and its cognate ligand are expressed on plasma membrane-derived extracellular vesicles. *Blood* (1998) 91:3862–74.
34. Andreola G, Rivoltini L, Castelli C, Huber V, Perego P, Deho P, et al. Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. *J Exp Med* (2002) 195:1303–16. doi: 10.1084/jem.20011624
35. Jeong WK, Wieckowski E, Taylor DD, Reichert TE, Watkins S, Whiteside TL. Fas ligand-positive membranous vesicles isolated from sera of patients with oral cancer induce apoptosis of activated T lymphocytes. *Clin Cancer Res* (2005) 11:1010–20. doi: 11/3/1010
36. Abusamra AJ, Zhong Z, Zheng X, Li M, Ichim TE, Chin JL, et al. Tumor exosomes expressing Fas ligand mediate CD8+ T-cell apoptosis. *Blood Cells Mol Dis* (2005) 35:169–73. doi: 10.1016/j.bcmd.2005.07.001
37. Huber V, Fais S, Iero M, Lugini L, Canese P, Squarcina P, et al. Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: Role in immune escape. *Gastroenterology* (2005) 128:1796–804. doi: 10.1053/j.gastro.2005.03.045
38. Liu C, Yu S, Zinn K, Wang J, Zhang L, Jia Y, et al. Murine Mammary Carcinoma Exosomes Promote Tumor Growth by Suppression of NK Cell Function. *J Immunol* (2006) 176:1375–85. doi: 10.4049/jimmunol.176.3.1375
39. Clayton A, Tabi Z. Exosomes and the MICA-NKG2D system in cancer. *Blood Cells Mol Dis* (2005) 34:206–13. doi: 10.1016/j.bcmd.2005.03.003
40. Clayton A, Mitchell JP, Court J, Linnane S, Mason MD, Tabi Z. Human Tumor-Derived Exosomes Down-Modulate NKG2D Expression. *J Immunol* (2008) 180:7249–58. doi: 10.4049/jimmunol.180.11.7249
41. Chalmin F, Ladoire S, Mignot G, Vincent J, Bruchard M, Remy-Martin JP, et al. Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells. *J Clin Invest* (2010) 120:457–71. doi: 10.1172/JCI40483
42. Xiang X, Poliakov A, Liu C, Liu Y, Bin DZ, Wang J, et al. Induction of myeloid-derived suppressor cells by tumor exosomes. *Int J Cancer* (2009) 124:2621–33. doi: 10.1002/ijc.24249
43. Yu S, Liu C, Su K, Wang J, Liu Y, Zhang L, et al. Tumor Exosomes Inhibit Differentiation of Bone Marrow Dendritic Cells. *J Immunol* (2007) 178:6867–75. doi: 10.4049/jimmunol.178.11.6867
44. Szajnik M, Czystowska M, Szczepanski MJ, Mandapathil M, Whiteside TL. Tumor-derived microvesicles induce, expand and up-regulate biological activities of human regulatory T cells (Treg). *PLoS One* (2010) 5:1–13. doi: 10.1371/journal.pone.0011469
45. Wang G-J, Liu Y, Qin A, Shah SV, Deng Z-b, Xiang X, et al. Thymus Exosomes-Like Particles Induce Regulatory T Cells. *J Immunol* (2008) 181:5242–8. doi: 10.4049/jimmunol.181.8.5242
46. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol* (2001) 22:633–40. doi: 10.1016/S1471-4906(01)02060-9
47. Geary CD, Sun JC. Memory responses of natural killer cells. *Semin Immunol* (2017) 31:11–9. doi: 10.1016/j.smim.2017.08.012
48. Van Audenaerde JRM, Roeyen G, Darcy PK, Kershaw MH, Peeters M, Smits ELJ. Natural killer cells and their therapeutic role in pancreatic cancer: A systematic review. *Pharmacol Ther* (2018) 189:31–44. doi: 10.1016/j.pharmthera.2018.04.003
49. Schauer IG, Zhang J, Xing Z, Guo X, Mercado-Uribe I, Sood AK, et al. Interleukin-1β promotes ovarian tumorigenesis through a p53/NF-κB-mediated inflammatory response in stromal fibroblasts. *Neoplasia (United States)* (2013) 15:409–20. doi: 10.1593/neo.121228
50. Xu B, Bai B, Sha S, Yu P, An Y, Wang S, et al. Interleukin-1β induces autophagy by affecting calcium homeostasis and trypsinogen activation in pancreatic acinar cells. *Int J Clin Exp Pathol* (2014) 7:3620–31.
51. Baginska J, Viry E, Berchem G, Poli A, Noman MZ, Van Moer K, et al. Granzyme B degradation by autophagy decreases tumor cell susceptibility to natural killer-mediated lysis under hypoxia. *Proc Natl Acad Sci U S A* (2013) 110:17450–5. doi: 10.1073/pnas.1304790110
52. Viry E, Baginska J, Berchem G, Noman MZ, Medves S, Chouaib S, et al. Autophagic degradation of GZMB/granzyme B :A new mechanism of hypoxic tumor cell escape from natural killer cell-mediated lysis. *Autophagy* (2014) 10:173–5. doi: 10.4161/auto.26924
53. Von Ahrens D, Bhagat TD, Nagrath D, Maitra A, Verma A. The role of stromal cancer-associated fibroblasts in pancreatic cancer. *J Hematol Oncol* (2017) 10:76. doi: 10.1186/s13045-017-0448-5
54. Richards KE, Zeleniak AE, Fishel ML, Wu J, Littlepage LE, Hill R. Cancer-associated fibroblast exosomes regulate survival and proliferation of pancreatic cancer cells. *Oncogene* (2017) 36:1770–8. doi: 10.1038/onc.2016.353
55. Fang Y, Zhou W, Rong Y, Kuang T, Xu X, Wu W, et al. Exosomal miRNA-106b from cancer-associated fibroblast promotes gemcitabine resistance in pancreatic cancer. *Exp Cell Res* (2019) 383:111543. doi: 10.1016/j.yexcr.2019.111543
56. Wu DM, Wen X, Han XR, Wang S, Wang YJ, Shen M, et al. Bone Marrow Mesenchymal Stem Cell-Derived Exosomal MicroRNA-126-3p Inhibits Pancreatic Cancer Development by Targeting ADAM9. *Mol Ther - Nucleic Acids* (2019) 16:229–45. doi: 10.1016/j.omtn.2019.02.022
57. Shang S, Wang J, Chen S, Tian R, Zeng H, Wang L, et al. Exosomal miRNA-1231 derived from bone marrow mesenchymal stem cells inhibits the activity of pancreatic cancer. *Cancer Med* (2019) 8:7728–40. doi: 10.1002/cam4.2633
58. Han S, Gonzalo DH, Feely M, Rinaldi C, Belsare S, Zhai H, et al. Stroma-derived extracellular vesicles deliver tumor-suppressive miRNAs to pancreatic cancer cells. *Oncotarget* (2018) 9:5764–77. doi: 10.18632/oncotarget.23532
59. Dunn WA. Studies on the mechanisms of autophagy: Maturation of the autophagic vacuole. *J Cell Biol* (1990) 110:1935–45. doi: 10.1083/jcb.110.6.1923
60. Savina A, Vidal M, Colombo MI. The exosome pathway in K562 cells is regulated by Rab11. *J Cell Sci* (2002) 115:2505–15. doi: 10.1083/jcb.149.1.1/a
61. Savina A, Furlán M, Vidal M, Colombo MI. Exosome release is regulated by a calcium-dependent mechanism in K562 cells. *J Biol Chem* (2003) 278:20083–90. doi: 10.1074/jbc.M301642200
62. Fader CM, Sánchez DG, Mestre MB, Colombo MI. TI-VAMP/VAMP7 and VAMP3/cellubrevin: two v-SNARE proteins involved in specific steps of the autophagy/multivesicular body pathways. *Biochim Biophys Acta - Mol Cell Res* (2009) 1793:1901–16. doi: 10.1016/j.bbamcr.2009.09.011
63. Fader CM, Sánchez D, Furlán M, Colombo MI. Induction of autophagy promotes fusion of multivesicular bodies with autophagic vacuoles in K562 cells. *Traffic* (2008) 9:230–50. doi: 10.1111/j.1600-0854.2007.00677.x
64. Xu J, Camfield R, Gorski SM. The interplay between exosomes and autophagy-partners in crime. *J Cell Sci* (2018) 131:1–11. doi: 10.1242/jcs.215210
65. Hessvik NP, Øverbye A, Brech A, Torgersen ML, Jakobsen IS, Sandvig K, et al. PIKfyve inhibition increases exosome release and induces secretory autophagy. *Cell Mol Life Sci* (2016) 73:4717–37. doi: 10.1007/s00018-016-2309-8
66. Alvarez-Erviti L, Seow Y, Schapira AH, Gardiner C, Sargent IL, Wood MJA, et al. Lysosomal dysfunction increases exosome-mediated alpha-synuclein release and transmission. *Neurobiol Dis* (2011) 42:360–7. doi: 10.1016/j.nbd.2011.01.029
67. Guo H, Chitiprolu M, Roncevic L, Javalet C, Hemming FJ, Trung MT, et al. Atg5 Disassociates the V1V0-ATPase to Promote Exosome Production and Tumor Metastasis Independent of Canonical Macroautophagy. *Dev Cell* (2017) 43:716–30. doi: 10.1016/j.devcel.2017.11.018
68. Murrow L, Malhotra R, Debnath J. ATG12-ATG3 interacts with Alix to promote basal autophagic flux and late endosome function. *Nat Cell Biol* (2015) 17:300–10. doi: 10.1038/ncb3112
69. Patton MC, Zubair H, Khan MA, Singh S, Singh AP. Hypoxia alters the release and size distribution of extracellular vesicles in pancreatic cancer cells to support their adaptive survival. *J Cell Biochem* (2020) 121:828–39. doi: 10.1002/jcb.29328
70. Varsano T, Dong M-Q, Niesman I, Gacula H, Lou X, Ma T, et al. GIPC Is Recruited by APPL to Peripheral TrkA Endosomes and Regulates TrkA Trafficking and Signaling. *Mol Cell Biol* (2006) 26:8942–52. doi: 10.1128/mcb.00305-06
71. Naccache SN, Hasson T, Horowitz A. Binding of internalized receptors to the PDZ domain of GIPC/synectin recruits myosin VI to endocytic vesicles.

Proc Natl Acad Sci U S A (2006) 103:12735–40. doi: 10.1073/pnas.0605317103
72. Varsano T, Taupin V, Guo L, Baterina OY, Farquhar MG. The PDZ Protein GIPC Regulates Trafficking of the LPA1 Receptor from APPL Signaling Endosomes and Attenuates the Cell's Response to LPA. *PLoS One* (2012) 7: e49227. doi: 10.1371/journal.pone.0049227
73. Wang LH, Kalb RG, Strittmatter SM, Nakamura F. Tanaka M. A PDZ protein regulates the distribution of the transmembrane semaphorin, M-SemF. *J Biol Chem* (1999) 274:14137–46. doi: 10.1074/jbc.274.20.14137
74. Bhattacharya S, Pal K, Sharma AK, Dutta SK, Lau JS, Yan IK, et al. GAIP interacting protein C-Terminus regulates autophagy and exosome biogenesis of pancreatic cancer through metabolic pathways. *PLoS One* (2014) 9:e114409. doi: 10.1371/journal.pone.0114409
75. Jin H, Liu P, Wu Y, Meng X, Wu M, Han J, et al. Exosomal zinc transporter ZIP4 promotes cancer growth and is a novel diagnostic biomarker for pancreatic cancer. *Cancer Sci* (2018) 109:2946–56. doi: 10.1111/cas.13737
76. Milane L, Singh A, Mattheolabakis G, Suresh M, Amiji MM. Exosome mediated communication within the tumor microenvironment. *J Control Release* (2015) 10:278–94. doi: 10.1016/j.jconrel.2015.06.029
77. Wu M, Tan X, Liu P, Yang Y, Huang Y, Liu X, et al. Role of exosomal microRNA-125b-5p in conferring the metastatic phenotype among pancreatic cancer cells with different potential of metastasis. *Life Sci* (2020) 255:117857. doi: 10.1016/j.lfs.2020.117857
78. Mingzhe Li, Huahu G, Wang Qi, Chen K, Marko K, Xiaodong Tian YY. Pancreatic Stellate Cells Derived Exosomal miR-5703 Promotes Pancreatic Cancer by Downregulating CMTM4 and Activating PI3K/Akt Pathway. *Cancer Lett* (2020) S0304-3835:30339–6. doi: 10.1016/j.canlet.2020.06.009
79. Comprehensive N and Network C. NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®) Pancreatic Adenocarcinoma. (2020).
80. Patel GK, Khan MA, Bhardwaj A, Srivastava SK, Zubair H, Patton MC, et al. Exosomes confer chemoresistance to pancreatic cancer cells by promoting ROS detoxification and miR-155-mediated suppression of key gemcitabine-metabolising enzyme, DCK. *Br J Cancer* (2017) 116:609–19. doi: 10.1038/bjc.2017.18
81. Mikamori M, Yamada D, Eguchi H, Hasegawa S, Kishimoto T, Tomimaru Y, et al. MicroRNA-155 controls exosome synthesis and promotes gemcitabine resistance in pancreatic ductal adenocarcinoma. *Sci Rep* (2017) 15:42339. doi: 10.1038/srep42339
82. de Jong OG, Verhaar MC, Chen Y, Vader P, Gremmels H, Posthuma G, et al. Cellular stress conditions are reflected in the protein and RNA content of endothelial cell-derived exosomes. *J Extracell Vesicles* (2012) 1:1–12. doi: 10.3402/jev.v1i0.18396
83. Noman MZ, Messai Y, Carré T, Akalay I, Méron M, Janji B, et al. Microenvironmental hypoxia orchestrating the cell stroma cross talk, tumor progression and antitumor response. *Crit Rev Immunol* (2011) 31:357–77. doi: 10.1615/CritRevImmunol.v31.i5.10
84. King HW, Michael MZ, Gleadle JM, Théry C, Zitvogel L, Amigorena S, et al. Hypoxic enhancement of exosome release by breast cancer cells. *BMC Cancer* (2012) 12:421. doi: 10.1186/1471-2407-12-421
85. Salomon C, Ryan J, Sobrevia L, Kobayashi M, Ashman K, Mitchell M, et al. Exosomal Signaling during Hypoxia Mediates Microvascular Endothelial Cell Migration and Vasculogenesis. *PLoS One* (2013) 8:1–24. doi: 10.1371/journal.pone.0068451
86. Park JE, Tan HS, Datta A, Lai RC, Zhang H, Meng W, et al. Hypoxic Tumor Cell Modulates Its Microenvironment to Enhance Angiogenic and Metastatic Potential by Secretion of Proteins and Exosomes. *Mol Cell Proteomics* (2010) 9:1085–99. doi: 10.1074/mcp.M900381-MCP200
87. Parolini I, Federici C, Raggi C, Lugini L, Palleschi S, De Milito A, et al. Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J Biol Chem* (2009) 284:34211–22. doi: 10.1074/jbc.M109.041152
88. Shen T, Jia S, Ding G, Ping D, Zhou L, Zhou S, et al. BxPC-3-Derived Small Extracellular Vesicles Induce FOXP3+ Treg through ATM-AMPK-Sirtuins-Mediated FOXOs Nuclear Translocations. *iScience* (2020) 23:101431. doi: 10.1016/j.isci.2020.101431
89. Wang X, Luo G, Zhang K, Cao J, Huang C, Jiang T, et al. Hypoxic tumor-derived exosomal miR-301a mediates M2 macrophage polarization via PTEN/PI3Kg to promote pancreatic cancer metastasis. *Cancer Res* (2018) 78:4586–98. doi: 10.1158/0008-5472.CAN-17-3841
90. Yue S, Mu W, Erb U, Zöller M. The tetraspanins CD151 and Tspan8 are essential exosome components for the crosstalk between cancer initiating cells and their surrounding. *Oncotarget* (2015) 6:2366–84. doi: 10.18632/oncotarget.2958
91. Lux A, Kahlert C, Grützmann R, Pilarsky C. c-Met and PD-l1 on circulating exosomes as diagnostic and prognostic markers for pancreatic cancer. *Int J Mol Sci* (2019) 20:3305. doi: 10.3390/ijms20133305
92. Trusolino L, Bertotti A, Comoglio PM. MET signalling: Principles and functions in development, organ regeneration and cancer. *Nat Rev Mol Cell Biol* (2010) 11:834–48. doi: 10.1038/nrm3012
93. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* (2000) 192:1027–34. doi: 10.1084/jem.192.7.1027
94. Smyth MJ, Teng MWL, Swann J, Kyparissoudis K, Godfrey DI, Hayakawa Y. CD4 + CD25 + T Regulatory Cells Suppress NK Cell-Mediated Immunotherapy of Cancer. *J Immunol* (2006) 176:1582–7. doi: 10.4049/jimmunol.176.3.1582
95. Tanaka A, Sakaguchi S. Regulatory T cells in cancer immunotherapy. *Cell Res* (2017) 27:109–18. doi: 10.1038/cr.2016.151
96. Birge RB, Boeltz S, Kumar S, Carlson J, Wanderley J, Calianese D, et al. Phosphatidylserine is a global immunosuppressive signal in efferocytosis, infectious disease, and cancer. *Cell Death Differ* (2016) 23:962–78. doi: 10.1038/cdd.2016.11
97. Lugini L, Cecchetti S, Huber V, Luciani F, Macchia G, Spadaro F, et al. Immune Surveillance Properties of Human NK Cell-Derived Exosomes. *J Immunol* (2012) 189:2833–42. doi: 10.4049/jimmunol.1101988
98. Jong AY, Wu CH, Li J, Sun J, Fabbri M, Wayne AS, et al. Large-scale isolation and cytotoxicity of extracellular vesicles derived from activated human natural killer cells. *J Extracell Vesicles* (2017) 6:1294368. doi: 10.1080/20013078.2017.1294368
99. Sun H, Shi K, Qi K, Kong H, Zhang J, Dai S, et al. Natural Killer Cell-Derived Exosomal miR-3607-3p Inhibits Pancreatic Cancer Progression by Targeting IL-26. *Front Immunol* (2019) 10:2819. doi: 10.3389/fimmu.2019.02819
100. You W, Tang Q, Zhang C, Wu J, Gu C, Wu Z, et al. IL-26 Promotes the Proliferation and Survival of Human Gastric Cancer Cells by Regulating the Balance of STAT1 and STAT3 Activation. *PLoS One* (2013) 8:e63588. doi: 10.1371/journal.pone.0063588
101. Rodella L, Zamai L, Rezzani R, Artico M, Peri G, Falconi M, et al. Interleukin 2 and interleukin 15 differentially predispose natural killer cells to apoptosis mediated by endothelial and tumour cells. *Br J Haematol* (2001) 115:442–50. doi: 10.1046/j.1365-2141.2001.03055.x

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Papademetrio, Garcia, Grasso and Alvarez. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Oncology

REVIEW
published: 25 February 2021
doi: 10.3389/fonc.2021.626309

Targeting Cytoprotective Autophagy to Enhance Anticancer Therapies

Malina Xiao[1†], Alice Benoit[1†], Meriem Hasmim[1], Caroline Duhem[2], Guillaume Vogin[3,4], Guy Berchem[1,2], Muhammad Zaeem Noman[1†] and Bassam Janji[1†]*

[1] Tumor Immunotherapy and Microenvironment (TIME) Group, Department of Oncology, Luxembourg Institute of Health (LIH), Luxembourg City, Luxembourg, [2] Department of Hemato-oncology, Centre Hospitalier du Luxembourg, Luxembourg City, Luxembourg, [3] Université de Lorraine - UMR 7365, Ingénierie Moléculaire et Physiopathologie Articulaire (IMoPA), Vandoeuvre-lès-Nancy, France, [4] Centre François Baclesse, Esch-sur-Alzette, Luxembourg

Autophagy is a highly regulated multi-step process that occurs at the basal level in almost all cells. Although the deregulation of the autophagy process has been described in several pathologies, the role of autophagy in cancer as a cytoprotective mechanism is currently well established and supported by experimental and clinical evidence. Our understanding of the molecular mechanism of the autophagy process has largely contributed to defining how we can harness this process to improve the benefit of cancer therapies. While the role of autophagy in tumor resistance to chemotherapy is extensively documented, emerging data point toward autophagy as a mechanism of cancer resistance to radiotherapy, targeted therapy, and immunotherapy. Therefore, manipulating autophagy has emerged as a promising strategy to overcome tumor resistance to various anti-cancer therapies, and autophagy modulators are currently evaluated in combination therapies in several clinical trials. In this review, we will summarize our current knowledge of the impact of genetically and pharmacologically modulating autophagy genes and proteins, involved in the different steps of the autophagy process, on the therapeutic benefit of various cancer therapies. We will also briefly discuss the challenges and limitations to developing potent and selective autophagy inhibitors that could be used in ongoing clinical trials.

Keywords: autophagy, cancer resistance, chemotherapy, radiotherapy, targeted therapy, immunotherapy

OPEN ACCESS

Edited by:
Waleska Kerllen Martins, Universidade Anhanguera de São Paulo, Brazil

Reviewed by:
Keith R. Laderoute, SRI International, United States
Yi Ding, Geisinger Health System, United States

****Correspondence:***
Bassam Janji
bassam.janji@lih.lu

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *05 November 2020*
Accepted: *15 January 2021*
Published: *25 February 2021*

Citation:
Xiao M, Benoit A, Hasmim M, Duhem C, Vogin G, Berchem G, Noman MZ and Janji B (2021) Targeting Cytoprotective Autophagy to Enhance Anticancer Therapies. Front. Oncol. 11:626309. doi: 10.3389/fonc.2021.626309

INTRODUCTION

Macroautophagy (referred to as autophagy) was first described in 1966 as a cellular process that occurs at the basal level in all cells (1). Autophagy relies on the formation of double-membraned vesicles known as autophagosomes, leading to the degradation of their cargo, such as damaged proteins or organelles (2). The autophagy process involves more than thirteen autophagy-related (ATG) proteins and requires the following major steps: (i) Initiation, (ii) Nucleation, (iii) Maturation, and (iv) Fusion with lysosome for cargo degradation (**Figure 1**). Originally described as a bulk degradation process, autophagy is now described as a highly selective degradation mechanism for the recycling of cellular components. Autophagy can be activated as an adaptive cellular response to external stimuli such as hypoxia, starvation, and different cancer therapies and therefore considered as a cytoprotective mechanism (1, 3–5).

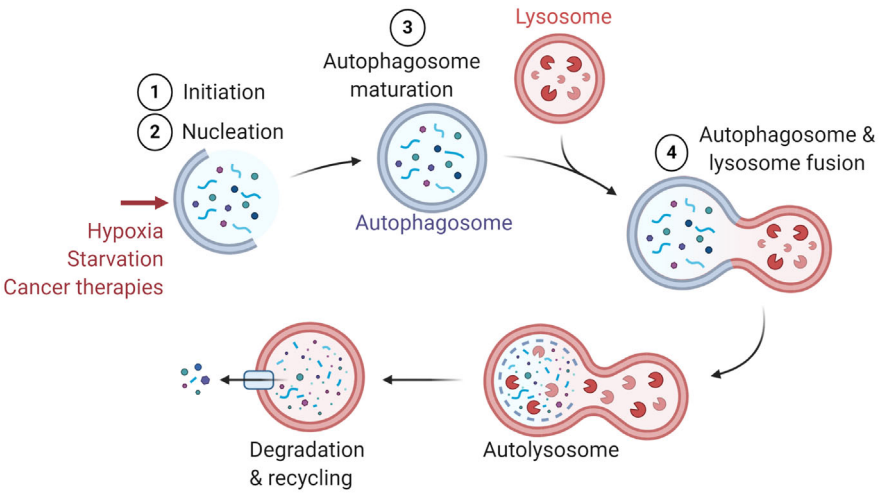


FIGURE 1 | General presentation of the major steps of autophagy. Several stimuli have been identified to induce autophagy such as hypoxia, starvation, and cancer therapies. The major steps of autophagy are: 1) Initiation, 2) Nucleation, 3) Maturation, and 4) Fusion with lysosome for the degradation and recycling of autophagosome constituents.

Autophagy is activated under nutrient deprivation or starvation condition, which resulted in a decrease of mTOR activity and an increase of Unc-51 like autophagy activating kinase 1 (ULK1) activation. Activated ULK1 is subsequently dissociated from the 5' adenosine monophosphate-activated protein kinase (AMPK), resulting in autophagy activation (6, 7). In addition to starvation, autophagy can also be activated in the tumor microenvironment by hypoxia through the hypoxia-inducible factor 1-α (HIF-α). The accumulation of HIF-1α in hypoxic cells activates the expression of BNIP/BNIP3L, which subsequently dissociates the complex between Bcl-2 and Beclin-1 (BECN1) to activate autophagy (8).

Autophagy was primarily considered as a tumor suppressive mechanism. Such a role was supported by studies showing that targeting BECN1, ATG5, and ATG7 promotes tumor initiation (9–11). In particular, evidences have demonstrated that Everolimus, an mTOR inhibitor and analogue of rapamycin, significantly increases mice survival in acute lymphoblastic leukemia in combination with Vincristine (12, 13). Conversely, many groups highlight the tumor supportive role of autophagy by showing its role in promoting tumor cell survival and growth in multiple tumor types (14, 15). The consensus appears to be that autophagy plays double-edged sword in suppressing tumor initiation and in promoting the survival of established tumors (16). Indeed, experimental evidence points at autophagy as a mechanism involved in cancer cell resistance to various therapies, such as chemotherapy, radiotherapy, targeted therapy, photodynamic therapy-induced apoptosis, and immunotherapy (17–21). Despite the complex interplay between the tumor suppressive and supportive role of autophagy in cancer (14), the vast majority of the clinical trials have focus on inhibiting autophagy with chloroquine (CQ) and hydroxychloroquine (HCQ) either alone or in combination with anticancer therapies (22). Therefore, autophagy inhibition has been suggested as a strategy to improve cancer therapies and has been considered in multiple clinical trials. Autophagy inhibitors have been classified according to their action on the major steps of autophagy and numerous preclinical studies have evaluated the therapeutic benefit of inhibiting autophagy.

In this review, we will summarize the impact of inhibiting the different steps of autophagy, either pharmacologically or genetically by silencing or knocking down autophagy-associated genes (**Figure 2**), and describe how autophagy can be leveraged to improve the therapeutic benefit of current cancer therapies and elicit a synergistic effect with antineoplastic agents.

IMPACT OF INHIBITING THE INITIATION AND NUCLEATION STEPS ON CANCER THERAPIES

The first step of the autophagy process, so-called the initiation step, involves the ULK protein complex including ATG13, ATG101/ULK1/2, and FIP200 (2, 23). The initiation step of autophagy facilitates the recruitment of the class III PI3K or VPS34 complex containing BECN1, VPS34, regulatory subunit 4 (VPS15/P150), activating molecule in BECN1-regulated autophagy protein 1 (AMBRA), UV radiation resistance-associated gene protein (UVRAG), BIF1, and ATG14L (2, 23) to the newly formed "phagophore". The recruitment of the class III PI3K constitutes the nucleation step. In this section, we will summarize the different drugs and/or strategies used to target the initiation and nucleation steps and briefly discuss data available on their efficacy in pre-clinical tumor mouse models.

Inhibition of ULK1

Several long noncoding RNAs (lncRNAs) have been reported to induce tumor chemoresistance to 5-fluorouracil (5-FU) such as

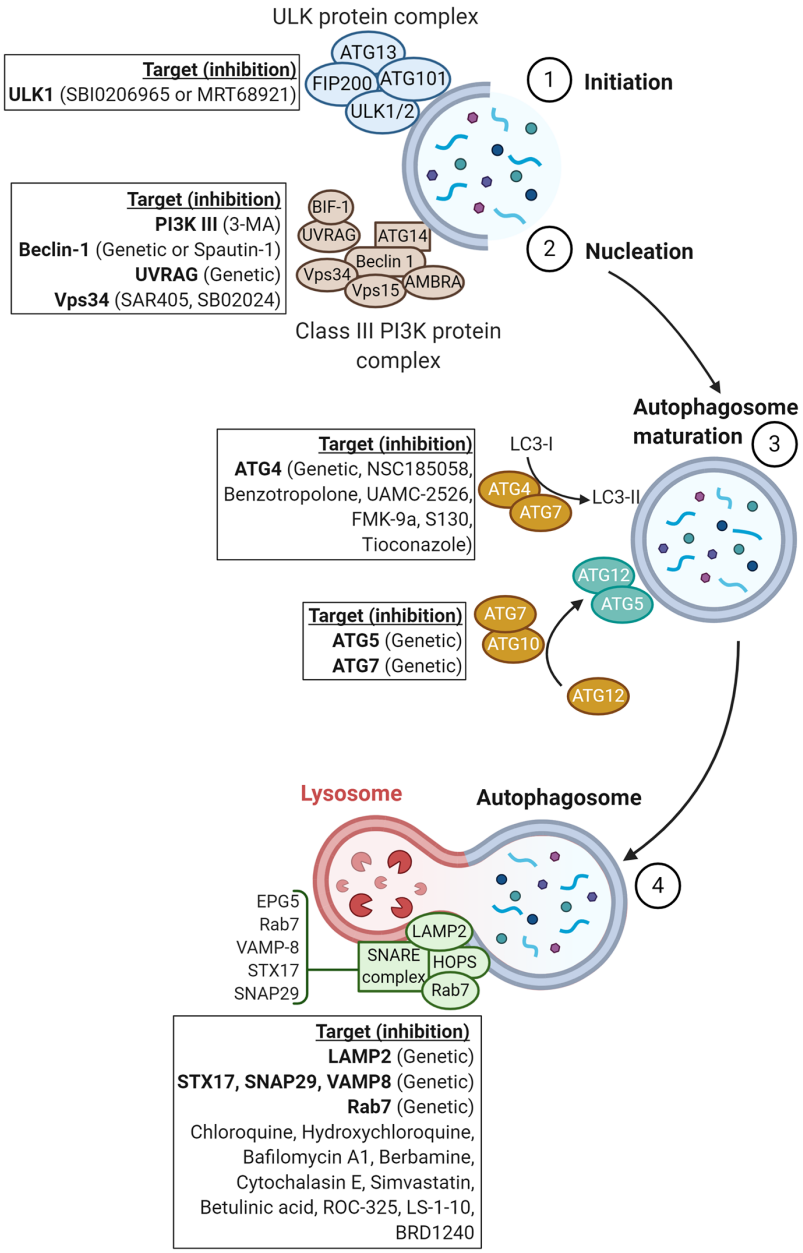


FIGURE 2 | Schematic representation of proteins involved in the major steps of autophagy. Genetic or pharmacological approaches targeting proteins involved in each step of autophagy are reported in squares.

lncRNA H19 and lncRNA small nucleolar RNA host gene 6 (SNHG6) in colorectal cancer (24, 25). SNHG6 promotes resistance of mice bearing RKO colon tumors to 5-FU. Furthermore, investigations showed that SNHG6 induced ULK1-dependent autophagy *via* sponging miR-26a-5p (25).

SBI0206965 is a highly selective inhibitor of ULK1 kinase (26) and it has been reported to sensitize NSCLC cells and acute myeloid leukemia (AML) cells to cisplatin- and daunorubicin-based chemotherapy, respectively, by decreasing cancer cell viability (27, 28). In pancreatic ductal adenocarcinoma (PDAC) cells, combining extracellular signal-regulated kinase (ERK) inhibitors with inhibitors of ULK1 complex (SBI0206965 or MRT68921) or with spautin-1, a specific inhibitor of two ubiquitin-specific peptidases USP10 and USP13 that control BECN1 degradation (29), decreased cell proliferation relative to ERK inhibitors alone (30). Recently, Chen et al. demonstrated that simultaneous inhibition of ULK1 (MRT68921) with NUAK1 (also known as ARK1) induces apoptosis in various cancer types (31).

Inhibition of Class III PI3K

Pre-treatment with class III PI3K inhibitors, such as 3-Methyladenine (3-MA), showed a significant improvement of the sensitivity of MDA-MB-231 and HBL-100 breast cancer cells to ionizing radiation (IR) despite an apparent low level of basal autophagy in HBL-100 cells (32). The therapeutic benefit of combining IR and 3-MA was observed in a xenograft esophageal squamous cell carcinoma mice model *in vivo* with a significant decrease in tumor volume relative to single treatment (33). Moreover, it has been demonstrated that 3-MA in combination with docetaxel, a semi-synthetic analog of paclitaxel, overcame docetaxel-induced autophagy and improved the sensitivity of lung adenocarcinoma (LAD) cells to docetaxel. Docetaxel-induced autophagy was mediated by High-mobility group box 1 (HMGB1) translocation, which participates in the BECN1 PI3K-III core complex formation *via* MEK/ERK1/2 pathway. Indeed, knockdown of *HMGB1* reverted the sensitivity of LAD cells to docetaxel (34).

Sorafenib is a well-known anti-angiogenic agent and remains the standard treatment in advanced unresectable hepatocellular carcinoma (HCC) (35). Over the past decade, several studies have focused on the underlying mechanisms induced by Sorafenib and exploring new combination therapies. In various types of cancers, Sorafenib has been described as inducing autophagy (36). Yuan et al. showed that 3-MA treatment in combination with Sorafenib significantly improved growth inhibition in HepG2, Hep3B, and PLC/PRF/5 treated cells (37). In triple-negative breast cancer (TNBC) cells, dual inhibition of autophagy and the Insulin-like growth factor (IGF) signaling pathway using 3-MA and NVP-AEW541, respectively, enhanced the NVP-AEW541-induced cell growth inhibition and apoptosis (38). In addition, preclinical studies have focused on exploring the benefit of epidermal growth factor receptor (EGFR) inhibitors such as gefitinib in EGFR-positive cancers (39). In TNBC cells, an increasing concentration of gefitinib combined with 3-MA significantly decreased cell viability *in vitro*. Interestingly, in TNBC xenograft mice models, a gefitinib and 3-MA combination resulted in a significant decrease in tumor volume and tumor weight compared to the gefitinib treated group. Further investigations revealed that autophagy inhibition by 3-MA enhanced gefitinib-induced GO/G1 cell cycle arrest, DNA damage, and cell death *via* the mitochondrial apoptosis pathway (40).

Selective VPS34 kinase inhibitors have acquired great interest as potential potent drugs to inhibit early autophagy. The VPS34 kinase inhibitor SAR405 in combination with everolimus, a well-known mTOR inhibitor approved for the treatment of various tumors (41), induced efficient autophagy inhibition and reduced renal tumor cell proliferation *in vitro* (42). In addition, the VPS34 kinase inhibitor SB02024 in combination with sunitinib, a tyrosine kinase inhibitor, significantly decreases cell viability and multicellular spheroid (MCS) growth in both MCF-7 and MDA-MB-231 breast cancer cell lines. Notably, inhibition of MCS growth was not observed with a chloroquine (CQ) and sunitinib combination (43). We have recently shown that pharmacological targeting of VPS34 kinase activity by SB02024 (Sprint Bioscience) or SAR405 (Sanofi) significantly decreased tumor growth and improved mice survival in melanoma B16-F10 and colorectal CT26 tumor mouse models (44). We provided evidence that deep changes in the immune landscape occurred in B16-F10 and CT26 mice models treated with VPS34 inhibitors (SB02024 and SAR405), characterized by increased infiltration of immune effectors such as NK, dendritic cells (DCs), M1 macrophages, and CD8+ T cells in the tumor microenvironment. Because there was no difference in the growth of tumors engrafted in NOD scid gamma (NSG) mice upon treatment with VPS34 inhibitors, these data clearly indicated that the tumor inhibitory effect of VPS34 inhibitors involves the host immune system. Moreover, we demonstrated that pro-inflammatory chemokines such as CCL5 and CXCL10 are responsible for NK and CD8+ T cell recruitment in B16-F10 tumors and CT26 tumors treated with VPS34 inhibitors relative to control. Interestingly, SB02024 or SAR405 improved the therapeutic benefit of anti-PD-1/PD-L1 by significantly reducing tumor growth and tumor weight and improving mice survival in B16-F10 and CT26 tumors (44).

Inhibition of Beclin-1

It has been reported that genetic inhibition of *BECN1* or *UVRAG* potentiated IR-induced DNA double-strand breaks (DSBs) and cell death in colorectal cancer cells (45). Furthermore, gene silencing of *BECN-1* enhanced the efficiency of fasudil (a RhoA/ROCK inhibitor) to induce apoptosis in esophageal squamous cell carcinoma cells (46). Similarly, *in vitro* suppression of *BECN1* reduces paclitaxel-mediated cell viability, colony formation, and induced apoptotic death in BT-474 and MDA-MB-231 breast cancer cells in dose- and time-dependent manners (47). Similar effects were observed in non-small cancer lung cancer (NSCLC) cells, endometrial carcinoma, nasopharyngeal carcinoma cells, and ovarian and renal cancers (48–53). Interestingly, the therapeutic benefit of paclitaxel was increased in a *Becn1*-targeted BT-474 xenograft mice model based on cleaved caspase-3 positive cells and inhibition of tumor growth (47).

In human chronic myeloid leukemia (CML) cells, co-treatment with spautin-1 and imatinib, a BCR-ABL tyrosine kinase inhibitor, potentiated imatinib-induced CML cell apoptosis in both the K562 cell line and primary cells (54). In line with this latter study, it has also been reported that imatinib in combination with microRNA-30a, identified as potent inhibitor of *BECN1* and *ATG5*, significantly increased the imatinib-mediated cytotoxicity in CML cells (55).

Tamoxifen is one of the most efficient endocrine treatments in estrogen receptor (ER) positive breast cancers, which account for 70% of the breast cancer subtypes. However, the therapeutic benefit of Tamoxifen is negatively impacted by the development of drug resistance (56). Gu et al. discovered that tamoxifen resistance was associated with an increased BECN1 and human epidermal growth factor receptor 2 (HER2) expression in breast cancer cells. *BECN1* silencing enhanced the sensitivity of breast cancer cells to tamoxifen by reducing tumor cell proliferation, migration, and invasion capabilities. These data highlight a novel

role of BECN1 in HER2 regulation that contributes to tamoxifen resistance (57).

Using several ovarian cancer cells, Zhang et al. showed that cancer cell resistance to cisplatin relied on autophagy-dependent induction of nuclear accumbens-1 (NAC1). Indeed, targeting *NAC1* or autophagy, *via* 3-MA or *BECN1* silencing, enhanced ovarian cancer cell sensitivity to cisplatin (58). Similar results were reported in adenoid cystic carcinoma of the salivary glands, glioma, and urothelial carcinoma (59–61).

Bevacizumab, a monoclonal anti-vascular endothelial growth factor (VEGF) antibody, is widely used to treat metastatic colorectal cancer, lung cancer and renal cell carcinoma (62–64). Knowing that bevacizumab induces autophagy, it has been reported that genetic inhibition of *BECN1* improves the anticancer effects of this drug in colorectal cancer cells (65). Likewise, improved clinical response to trastuzumab was observed in HER2+ breast cancer displaying loss of *BECN1* gene (38, 66). The role of autophagy, including BECN1, in tumor resistance to targeted therapy is comprehensively reviewed by Mele et al. (67).

We have previously reported that genetic targeting of *Becn1* in melanoma cells prevents the degradation of Natural killer (NK)-derived Granzyme B and enhances melanoma susceptibility to NK-mediated killing (68). Furthermore, we showed that the infiltration of NK cells into *Becn1* defective melanoma is increased, which results in significant inhibition of tumor growth (69). Importantly, the impact of inhibiting autophagy on the infiltration of cytotoxic immune cells into the tumors and the decrease in tumor growth is also reported by other studies (70–73).

TARGETING AUTOPHAGOSOME MATURATION GENES ATG4B, ATG5, AND ATG7 POTENTIATES ANTICANCER THERAPIES

The third major step of autophagy involves two key complexes that promote the expansion of the phagophore membrane and result in the formation of a double-membraned vesicle named autophagosome. The first complex involves cooperation between ATG4B and ATG7, allowing for the conjugation of LC3I with phosphatidylethanolamine (PE) to form LC3II. LC3II is subsequently incorporated into the newly formed autophagosome (74). The second complex includes ATG7 and the E2-like enzyme ATG10, which are involved in ATG5-ATG12 conjugation (2). In this section, we will describe the therapeutic benefit of inhibiting autophagy genes involved in the maturation of autophagosomes.

Inhibition of ATG4B

The serine/threonine protein kinase MST4, also known as mammalian STE20-like protein kinase 4 (MST4) (75), was reported to facilitate p-ERK pathway and promote epithelial to mesenchymal transition (EMT) and cancer metastasis in gastric cancer (76). MST4 is associated with prostate cancer, hepatocellular carcinoma, and breast cancer progression (77, 78). It has been reported that MST4 directly phosphorylates ATG4B at S383 position (79). Furthermore, ATG4B inhibition, by NSC185058 (80), improves the anti-tumor effect of radiotherapy in intracranial glioblastoma (GBM) patient derived xenograft (PDX) mice models (79). These data suggest a potential interconnection between MST4, autophagy and malignancy in GBM; however, the value of direct targeting MST4 as a strategy to modulate autophagy remains to be defined.

Recently, benzotropolone derivatives were synthetized and tested for ATG4B inhibition. UAMC-2526 was selected as the best candidate according to its efficiency to reduce basal autophagy and its high stability in the plasma. A combination of UAMC-2526 with oxaliplatin-based chemotherapy reduced colorectal cell proliferation and promoted tumor growth inhibition in HT29 colorectal tumor-bearing mice (81).

FMK-9a is another ATG4B inhibitor, reported to attenuate the pro-LC3 cleavage process and the LC3-PE delipidation. FMK-9a could also induce autophagy independent of its inhibition on ATG4B activity (82). Recently, S130 has been identified to bind and inhibit ATG4B, hence attenuating the delipidation of LC3-II and suppressing the recycling of LC3-I in colorectal cancer cells. Therefore, S130 has been described as a novel small-molecule to improve cancer therapy (83).

Moreover, an FDA-approved drug screening identified tioconazole as a new ATG4 inhibitor. Tioconazole treatment enhanced doxorubicin efficiency by decreasing cell viability in H4, HCT116, and MDA-MB-231 cells. Interestingly, the combination of tioconazole and doxorubicin showed an enhanced antitumor effect in HCT116 xenografted mice relative to each drug alone (84). In this context, it has been reported that the HER2 status was positively correlated with the expression of ATG4B protein. Interestingly, ATG4B silencing was associated with reduced viability of trastuzumab treated HER2+ cells compared to trastuzumab treatment alone (85).

Inhibition of ATG5 and ATG7

In A549 human lung cancer, overcoming the cytoprotective effect of autophagy induced by cisplatin, *via ATG5* silencing, improves cancer cell apoptosis, as compared to cisplatin treatment alone (86). Dual combination of Epirubicin, a structural analog of doxorubicin, with *ATG5*- or *ATG7*-silencing, significantly reduced cell viability in anthracycline-sensitive and resistant TNBC cells (87). O'Donovan et al. showed that combining both siRNA *BECN1* and *ATG7* decreased cell survival in 5-FU-treated esophageal cancer cells while targeting *BECN1* or *ATG7* alone had no impact (88). Therefore, it appears that targeting different steps of autophagy may be a more appropriate strategy to improve chemotherapy efficacy. In TBNC cells, Wu et al. demonstrated that dual inhibition of *ATG7*, genetically, and IGF-1R pharmacologically, promotes apoptosis and cell growth inhibition (38). In PDAC cells, genetic inhibition of *ATG5* or *ATG7* significantly improved the effect of ERK inhibitors on inhibiting cell proliferation relative to ERK inhibitors alone (30). Dual combination of *ATG5* siRNA and docetaxel, a well-known second-line approved treatment in NSCLC, decreased cell proliferation together with increasing cytotoxicity and apoptosis in LAD cells (34). In renal cell

carcinoma cells, *ATG5* silencing or 3-MA treatment in combination with Sorafenib enhanced the sensitivity of RCC cells to Sorafenib (89).

IMPACT OF INHIBITING AUTOPHAGOSOME-LYSOSOME FUSION ON THE RESPONSE TO VARIOUS ANTICANCER THERAPIES

The final step of autophagy consists of fusion between autophagosomes and lysosomes for the degradation, and recycling of damaged proteins and organelles. Thus, the outer membrane of autophagosomes merges with the lysosomal membrane, and then the inner membrane is degraded (90). The principal factors involved in autophagosome-lysosome fusion are the homotypic fusion and protein sorting (HOPS) complex, RAB7, and the N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (91). Other proteins, such as the lysosomal-associated membrane protein 2 (LAMP2), are also required for proper fusion (92). This part will focus on the impact of targeting these factors and the fusion step on the therapeutic benefit of radiotherapy, chemotherapy, targeted therapy, and immunotherapy.

Targeting the Soluble N-Ethylmaleimide-Sensitive Factor Attachment Protein Receptors (SNAREs) to Enhance Response to Conventional Treatments

Syntaxin 17 (STX17), SNAP29, and vesicle-associated membrane protein 8 (VAMP8) are N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) involved in the autophagosome-lysosome fusion. During this process, STX17 is recruited to the outer membrane of autophagosomes through HOPS complex and interacts with VAMP8, located on lysosomes. This interaction is enhanced by SNAP29, which forms a SNARE complex with STX17 and VAMP8 (93). Knockdown of *STX17* causes a blockade of the fusion between autophagosomes and lysosomes and results in the accumulation of autophagosomes (93). Therefore, targeting SNARE proteins is considered a strategy for preventing the late step of autophagy. Moreover, SNAP29-STX17-VAMP8 complex formation can be enhanced by the down-regulation of *O*-GlcNAc transferase (OGT). This was correlated with the resistance of ovarian cancer to cisplatin treatment (94), and highlights that targeting SNAP29-STX17-VAMP8 complex by overexpression of OGT could improve the sensitivity to cisplatin treatment. Overexpression of *VAMP8* has also been associated with resistance to temozolomide in human glioma cells, and knockdown of *STX17* in glioma cells overexpressing *VAMP8* led to increased chemosensitivity (95).

In addition, Berbamine, a natural product isolated from traditional Chinese medicine, inhibits autophagosome-lysosome fusion by preventing the interaction between VAMP8 and SNAP29. Berbamine was proposed as a new potential inhibitor of autophagy that could enhance the effects of chemotherapy treatment (96). Moreover, Berbamine has been investigated as a potential anticancer drug in several studies and seems to act on multiple pathways such as MEK/ERK (97) and WNT/β-catenin pathways (98).

Targeting Lysosomal-Associated Membrane Protein 2 (LAMP2) as a Potential Target to Inhibit Autophagosome-Lysosome Fusion and Improve the Response to Anti-Cancer Therapies

LAMP2 is a glycosylated protein ubiquitously expressed, and mostly located on lysosome membranes. LAMP2 is required for the proper fusion between autophagosomes and lysosomes (92). In neuroendocrine prostate cancer, knockdown of *LAMP2* by siRNA induced an autophagy blockade and decreased both cancer cell proliferation and neuroendocrine markers. These results indicate that LAMP2 plays a dual role in cell survival, by inducing autophagy and in the differentiation of androgen-sensitive human prostate adenocarcinoma cells into neuroendocrine prostate cancer cells (99). In addition, a recent *in silico* approach showed that the expression of *LAMP2* was decreased in prostate cancer tissues as compared to normal prostate tissues (100), indicating that the expression level of *LAMP2* could act as a regulatory element in cancer progression. Another study compared the expression level of *LAMP2* in salivary adenoid cystic carcinoma and pleomorphic adenoma and/or a normal salivary gland (101). The results showed an increased expression of *LAMP2* in salivary adenoid cystic carcinoma, which was associated with cancer progression. Although the expression level of *LAMP2* seems to be different in various cancer types, several data suggest that *LAMP2* is a potential target for cancer therapy in combination with conventional treatments. This statement was supported by data showing that the silencing of *LAMP2* by siRNAs led to a radiosensitization of prostate cancer cell lines (102). In addition, a reduced expression of *LAMP2* has been associated with a decreased resistance to both cisplatin in human ovarian carcinoma cells (103) and azacitidine in acute myeloid leukemia (104). Nevertheless, the sensitization to radiotherapy and chemotherapy by *LAMP2* targeting should be investigated in other types of cancer cells.

Overexpression of *RAB7* as a Potential Strategy to Improve Sensitivity to Anti-Cancer Treatments

RAB7 is a small GTPase localized to late endosomes and lysosomes and has multiple functions in autophagy. In mammalian, RAB7 is not directly involved in the autophagosome-lysosome fusion but rather in autolysosome maturation under nutrient-rich conditions (105). The significance of RAB7 as a target for autophagy modulation is not well defined so far.

The role of RAB7 in cancer progression has recently been described as a protein involved in promoting the proliferation,

invasion, and migration of gastric cancer cells (106). RAB7 has also been associated with chemoresistance to cisplatin. Indeed, *RAB7* was downregulated in cisplatin-resistant cervical cancer cell lines as compared to chemosensitive ones (107). Additionally, *RAB7* overexpression induced chemosensitization of cisplatin-resistant cells, while depletion of *RAB7* by siRNA induced resistance to cisplatin in chemosensitive cells (108). Furthermore, RUBICON (Run domain Beclin-1 interacting and cysteine-rich containing), a negative regulator of autophagy, inhibits autophagosome-lysosome fusion and interacts with RAB7-GTP *via* a RUBICON homology (RH) domain (109).

Impact of Using Drugs Inhibiting the Last Step of Autophagy Process on Various Cancer Therapies

CQ and its derivate, hydroxychloroquine (HCQ), are the only drugs having shown their ability to block the last step of autophagy and being approved by the Food and Drug Administration (FDA) for clinical use. Indeed, CQ blocks the autophagic flux by altering autophagosome fusion with lysosomes, presumably by interfering with SNAP29 recruitment (110). In addition, CQ cytotoxicity induced autophagy-associated cell death associated with nuclei abnormalities, lipofuscinogenesis, and senescence (111). As reported in clinicaltrials.gov, CQ is currently being investigated as a potent anticancer drug in small cell lung cancer, breast cancer, pancreatic cancer, glioblastoma, melanoma, and other types of cancers.

Because CQ and HCQ are the only autophagy inhibitors available and approved for clinical use, multiple studies evaluated the potential effects of CQ *in vitro*, in combination with anticancer treatments. In fact, CQ was responsible for radio-sensitizing bladder cancer cells and bladder cancer xenografts in mice (112). CQ also had a synergetic effect with radiotherapy on glioma initiating cells by inducing apoptosis and inhibiting autophagy induced by ionizing radiation (113). The same synergistic effects were observed on glioblastoma cell lines (114). CQ administered after radiation is also capable of increasing the death of breast cancer cells and tumor regression *in vivo* (115). The use of CQ as a potent enhancer of radiotherapy is currently being evaluated in clinical trials involving small cell lung cancer (NCT01575782) and glioblastoma (NCT04397679), and in patients with brain metastases from solid tumors (NCT01894633). It should be highlighted that CQ sensitized various breast cancer cell lines to cisplatin and LY294002, reported to induce autophagy in these cells. However, CQ sensitization in these cells occurred independent of autophagy inhibition. Therefore, the autophagy independent sensitizing effects of CQ should be considered in clinical trials where CQ or its derivatives are used in the treatment of cancer (116, 117). In HCT-116 and HT-29 colorectal cancer cell lines, it has been reported that CQ sensitized these cell lines to radiation and 5-FU treatment and resulted in a significant decrease in clonogenic survival of HT-29 cell line without any impact on cell cycle progression or cell death (118). However, in GBM, CQ was found to induce P53-independent cell deaths that do not require caspase-mediated apoptosis. The CQ derivatives, Quinacrine and Mefloquine, are more potent and displayed superior blood-brain barrier penetration compared to CQ (119).

Maycotte et al. evaluated the effects of combining CQ with chemotherapeutic drugs such as the DNA damaging agent cisplatin, the mTOR inhibitor Rapamycin, and the PtdIns3K inhibitor LY294002 in two mouse breast cancer cell lines (117). While the combination of CQ and cisplatin had no significant effect on the viability of both cell lines, CQ combined with PtdIns3K and mTOR inhibition sensitized both cell lines. However, the CQ-mediated sensitization seems to be independent of autophagy, since this sensitization was not observed following *Atg12* and *Becn1* knockdown (117). A similar result was observed in KRAS-driven cancer cell lines where the antiproliferative effects of CQ were similar between *ATG7*-deficient tumor cell lines with undetectable autophagic flux and *ATG7*-efficient tumor cell lines (120). In addition, CQ sensitizes bladder cancer cells to cisplatin treatment by inhibiting cisplatin-induced autophagy (121). Similar results were observed in nasopharyngeal carcinoma cells (122) and hypopharyngeal squamous cell carcinoma xenografted mice (123). This suggests that CQ effects, in combination with chemotherapy, depend on the type of cancer and therefore require further investigation.

Several studies have been conducted on CQ in combination with targeted therapies. Erlotinib and Rapamycin are two tyrosine kinase inhibitors targeting the EGFR and the mammalian target of Rapamycin, respectively. These anticancer drugs are particularly used for NSCLC treatment where EGFR and PI3K/AKT/mTOR pathways are often dysregulated. It has been shown that the combination of Erlotinib and Rapamycin with Monensin, a polyether antibiotic inhibiting autophagosome-lysosome fusion, improved Erlotinib and Rapamycin induced tumor growth inhibition and apoptosis in NSCLC (124). Similar results were observed in prostate cancer cells by Monensin, although the involvement of autophagy inhibition was not clearly suggested in this study (125). Furthermore, the combination of the tyrosine kinase inhibitor, sunitinib, with CQ or *LAMP2* knockdown also showed promising results in a metastatic pancreatic neuroendocrine tumor mice model (126). The combination of the monoclonal antibody trastuzumab with CQ in HER2+ breast cancer (127) also led to promising results. Indeed, CQ sensitized both trastuzumab-resistant breast cancer cells and trastuzumab-resistant xenografts, resulting in increased cell death *in vitro* and decreased tumor growth *in vivo*.

In addition to its effects in combination with radiotherapy, chemotherapy, and targeted therapy, CQ seems to be responsible for various effects on the immune system. CQ resets tumor-associated M2 macrophages to the tumor-inhibiting M1 phenotype in B16 melanoma and H22 hepatocarcinoma mouse tumor models, and ameliorates the immunosuppressive tumor immune microenvironment through a lysosomal calcium-TFEB pathway (91). Another recent article showed that CQ in combination with 5-FU increased the driving of DC

maturation by HCT-116 colon cancer cells, and in this way stimulates T cell responses induced by tumor cell lysates (128). Considering the impact of CQ on the immune system, CQ was tested in combination with dual CTLA4 and PD-1 immune checkpoint blockade in orthotopic tumors established from pancreatic ductal adenocarcinoma cells. This study revealed an increased CD8+ T cell infiltration within the tumors and a tumor sensitization to anti-PD-1 and anti-CTLA4 therapy when combined with CQ (129). These data support further investigations on the potential effect of combining CQ with other immunotherapy such as new immune checkpoint inhibitors, T-cell transfer therapy, or monoclonal antibodies, on the immunosuppressive tumor microenvironment.

Besides CQ, Bafilomycin A1 (BafA1) is another drug acting at the last step of autophagy. BafA1 is an antibiotic targeting the vacuolar H+-ATPase enzyme, thus inducing acidification of lysosomal pH, therefore inhibiting lysosomal degradation capacity. BafA1 has also shown promising effects in combination with chemotherapy, since BafA1 increased cisplatin cytotoxicity in tongue squamous cell carcinoma cells and bladder cancer cells (121, 130). BafA1 also increased chemosensitivity to 5-FU in gastric cancer cells (131). Other drugs have been described to target autophagosome-lysosome fusion, such as LS-1-10, Cytochalasin E and Simvastatin. LS-1-10 is a novel acridine derivative sharing structure with CQ and HCQ. LS-1-10 has a dual function, it is able to both induce DNA damage and block autophagosome-lysosome fusion. In addition, LS-1-10 is significantly more potent in reducing cell viability than CQ (>50%) in colon cancer cells (132).

Cytochalasin E is a fungal toxin found in *Aspergillus clavatus*, which binds to actin filaments. Cytochalasin E can inhibit autophagosome-lysosome fusion in the same way as CQ. In addition, cytochalasin enhances the effect of bortezomib in human lung cancer cells (133). Simvastatin is a powerful inhibitor of hydroxymethylglutaryl CoA reductase, an enzyme involved in cholesterol biosynthesis. Interestingly, Simvastatin can induce cell death in astrocytoma, neuroblastoma, glioblastoma, breast cancer, and lung adenocarcinoma (134). Moreover, this drug appears to inhibit the fusion between autophagosomes and lysosomes and increase the effects of Temozolomide in glioblastoma cell lines (135). Finally, the combination of Simvastatin with Vorinostat, a histone deacetylase inhibitor, inhibits autophagy by preventing RAB7 prenylation and decreases tumor growth in mice bearing triple-negative breast tumors (136).

CONCLUSION

Despite the dual role of autophagy in suppressing tumor initiation and in promoting the survival of established tumors (16), the studies reported in this review highlight the pivotal role of autophagy as a cytoprotective and therapy resistance mechanism in cancer. Therefore, strategies used to modulate autophagy for enhancing the therapeutic benefit of current anticancer therapies are an area of intense investigation. Based on data reported in this review, we strongly believe that inhibiting autophagy represents a new paradigm for overcoming therapy resistance and enhancing drug sensitivity in multiple tumor cell types. Because autophagy inhibition is currently used in many clinical trials along with different therapeutic strategies, it is reasonable to consider that several cancer treatments themselves induce autophagy in tumor cells.

Among the various autophagy inhibitors, CQ or its derivative HCQ are the major drugs used in clinical trials, with mitigated success. While CQ provides promising results in combination with conventional anticancer therapies, in some studies CQ sensitization appears to be independent of autophagy inhibition. Therefore, more selective and potent autophagy inhibitors must be designed to definitively endorse the therapeutic benefit of targeting autophagy in cancer patients. While CQ and HCQ inhibits the last step of autophagy (110), other druggable autophagy proteins have recently been proposed, which include the early autophagy protein BECN1 (45) and its interacting protein VPS34 (or PI3K class III) (41, 42, 44). Thus, more potent and selective autophagy specific inhibitors are currently in pre-clinical development; these include drugs targeting earlier steps in the autophagy process, such as ULK1, VPS34, and ATG4B (27, 28) (79). Other factors interacting with the autophagy process could also be considered as potential targets to inhibit autophagy and overcome therapy resistance, such as AMPK and HIF-1α, which are reported as key inducers of autophagy through negative regulation of the mTOR pathways and inducing hypoxic conditions, respectively (6, 7, 137). Other drugs are able to target the late step of autophagy by interacting with lysosomes. ROC-325 has been described as a potent autophagy inhibitor exhibiting superior *in vitro* and *in vivo* anticancer effects compared to CQ. In Renal cell carcinoma RCC, ROC-325 induced an accumulation of autophagosomes *in vitro* and inhibited RCC growth and survival in an ATG5/7-dependent manner *in vivo* by disrupting autophagic degradation (138). In acute myeloid leukemia, ROC-325 improved the anti-leukemic activity of azacitidine through inhibiting autophagy (139). BRD1240 is a small-molecule suppressing the V-ATPase function and therefore inhibiting the lysosomal acidification property. It has been reported that, similar to BafA1, BRD1240 inhibited autolysosome formation and subsequently triggering a significant accumulation of autophagosomes (140). Similar to ROC-325 and LS-1-10, Betulinic acid (BA) disrupt the degradative lysosomal function (74, 141–143), leading to the accumulation of mitochondria inside dysfunctional autolysosomes. Such a lysosome-mitochondrial stress axis is responsible for the induction of lipofuscinogenesis and ageing (143).

It should be highlighted that even if a selective and potent autophagy inhibitor is identified, the challenging task is to demonstrate that the therapeutic benefit that could be observed is indeed related to inhibition of the autophagy process, as almost all autophagy-related genes have non-autophagic functions. Another challenge is to maintain a balance between the benefits gained by autophagy inhibition and the deleterious effects of this inhibition in cancer patients. Indeed, the process of autophagy seems to both activates and inhibits cellular senescence (144), and chronic inhibition of autophagy appears to increase permanently the risk of cancer (145). Finally, considering the controversial role of autophagy regarding its cytoprotective or cytotoxic function, it is more likely obvious that the clinical outcome of combination treatment between an inhibitor of autophagy with chemotherapy,

radiotherapy, targeted therapy, or immunotherapy would lead to contradictory or equivocal results (146). Therefore, the last challenge to overcome would be to determine whether patients would benefit from autophagy inhibition prior to conventional therapies, with for example the use of novel biomarkers of cytoprotective autophagy.

AUTHOR CONTRIBUTIONS

MX, AB, MH, CD, GV, GB, MN, and BJ have contributed in writing and editing the manuscript. BJ has generated figures. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by Luxembourg Institute of Health and grants from the Luxembourg National Research Fund (PRIDE15/10675146/CANBIO and C18/BM/12670304/COMBATIC); FNRS Televie (grants 7.4606.18 and 7.4579.20); Fondation Cancer, Luxembourg (FC/2018/06); Kriibskrank Kanner Foundation, Luxembourg (2019); RCMS foundation, and Action LIONS Vaincre le Cancer Luxembourg. The authors also declare that this study received funding from Janssen Cilag Pharma and Roche Pharma. These funders were not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

REFERENCES

1. De Duve C, Wattiaux R. Functions of lysosomes. *Annu Rev Physiol* (1966) 28:435–92. doi: 10.1146/annurev.ph.28.030166.002251
2. Levy JMM, Towers CG, Thorburn A. Targeting autophagy in cancer. *Nat Rev Cancer* (2017) 17(9):528–42. doi: 10.1038/nrc.2017.53
3. Noman MZ, Janji B, Kaminska B, Van Moer K, Pierson S, Przanowski P, et al. Blocking hypoxia-induced autophagy in tumors restores cytotoxic T-cell activity and promotes regression. *Cancer Res* (2011) 71(18):5976–86. doi: 10.1158/0008-5472.CAN-11-1094
4. Sui X, Chen R, Wang Z, Huang Z, Kong N, Zhang M, et al. Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. *Cell Death Dis* (2013) 4:e838. doi: 10.1038/cddis.2013.350
5. Palorini F, Cozzarini C, Gianolini S, Botti A, Carillo V, Iotti C, et al. First application of a pixel-wise analysis on bladder dose-surface maps in prostate cancer radiotherapy. *Radiother Oncol* (2016) 119(1):123–8. doi: 10.1016/j.radonc.2016.02.025
6. Jung CH, Ro SH, Cao J, Otto NM, Kim DH. mTOR regulation of autophagy. *FEBS Lett* (2010) 584(7):1287–95. doi: 10.1016/j.febslet.2010.01.017
7. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol* (2011) 13(2):132–41. doi: 10.1038/ncb2152
8. Bellot G, Garcia-Medina R, Gounon P, Chiche J, Roux D, Pouyssegur J, et al. Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. *Mol Cell Biol* (2009) 29(10):2570–81. doi: 10.1128/MCB.00166-09
9. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest* (2003) 112(12):1809–20. doi: 10.1172/JCI20039
10. Yue Z, Jin S, Yang C, Levine AJ, Heintz N. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc Natl Acad Sci USA* (2003) 100(25):15077–82. doi: 10.1073/pnas.2436255100
11. Takamura A, Komatsu M, Hara T, Sakamoto A, Kishi C, Waguri S, et al. Autophagy-deficient mice develop multiple liver tumors. *Genes Dev* (2011) 25(8):795–800. doi: 10.1101/gad.2016211
12. Crazzolara R, Cisterne A, Thien M, Hewson J, Baraz R, Bradstock KF, et al. Potentiating effects of RAD001 (Everolimus) on vincristine therapy in childhood acute lymphoblastic leukemia. *Blood* (2009) 113(14):3297–306. doi: 10.1182/blood-2008-02-137752
13. Singh SS, Vats S, Chia AY, Tan TZ, Deng S, Ong MS, et al. Dual role of autophagy in hallmarks of cancer. *Oncogene* (2018) 37(9):1142–58. doi: 10.1038/s41388-017-0046-6
14. Kimmelman AC. The dynamic nature of autophagy in cancer. *Genes Dev* (2011) 25(19):1999–2010. doi: 10.1101/gad.17558811
15. Guo JY, Xia B, White E. Autophagy-mediated tumor promotion. *Cell* (2013) 155(6):1216–9. doi: 10.1016/j.cell.2013.11.019
16. Janji B, Viry E, Moussay E, Paggetti J, Arakelian T, Mgrditchian T, et al. The multifaceted role of autophagy in tumor evasion from immune surveillance. *Oncotarget* (2016) 7(14):17591–607. doi: 10.18632/oncotarget.7540
17. Ito H, Daido S, Kanzawa T, Kondo S, Kondo Y. Radiation-induced autophagy is associated with LC3 and its inhibition sensitizes malignant glioma cells. *Int J Oncol* (2005) 26(5):1401–10. doi: 10.3892/ijo.26.5.1401
18. Amaravadi RK, Yu D, Lum JJ, Bui T, Christophorou MA, Evan GI, et al. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. *J Clin Invest* (2007) 117(2):326–36. doi: 10.1172/JCI28833
19. Ertmer A, Huber V, Gilch S, Yoshimori T, Erfle V, Duyster J, et al. The anticancer drug imatinib induces cellular autophagy. *Leukemia* (2007) 21 (5):936–42. doi: 10.1038/sj.leu.2404606
20. Wei MF, Chen MW, Chen KC, Lou PJ, Lin SY, Hung SC, et al. Autophagy promotes resistance to photodynamic therapy-induced apoptosis selectively in colorectal cancer stem-like cells. *Autophagy* (2014) 10(7):1179–92. doi: 10.4161/auto.28679
21. Janji B, Berchem G, Chouaib S. Targeting Autophagy in the Tumor Microenvironment: New Challenges and Opportunities for Regulating Tumor Immunity. *Front Immunol* (2018) 9:887. doi: 10.3389/fimmu.2018.00887
22. Towers CG, Thorburn A. Therapeutic Targeting of Autophagy. *EBioMedicine* (2016) 14:15–23. doi: 10.1016/j.ebiom.2016.10.034
23. Xiao M, Noman MZ, Menard L, Chevigne A, Szpakowska M, Bosseler M, et al. Driving Cytotoxic Natural Killer Cells into Melanoma: If CCL5 Plays the Music, Autophagy Calls the Shots. *Crit Rev Oncog* (2018) 23(5-6):321–32. doi: 10.1615/CritRevOncog.2018027526
24. Wang M, Han D, Yuan Z, Hu H, Zhao Z, Yang R, et al. Long non-coding RNA H19 confers 5-Fu resistance in colorectal cancer by promoting SIRT1-mediated autophagy. *Cell Death Dis* (2018) 9(12):1149. doi: 10.1038/s41419-018-1187-4
25. Wang X, Lan Z, He J, Lai Q, Yao X, Li Q, et al. LncRNA SNHG6 promotes chemoresistance through ULK1-induced autophagy by sponging miR-26a-5p in colorectal cancer cells. *Cancer Cell Int* (2019) 19:234. doi: 10.1186/s12935-019-0951-6
26. Egan DF, Chun MG, Vamos M, Zou H, Rong J, Miller CJ, et al. Small Molecule Inhibition of the Autophagy Kinase ULK1 and Identification of ULK1 Substrates. *Mol Cell* (2015) 59(2):285–97. doi: 10.1016/j.molcel.2015.05.031
27. Tang F, Hu P, Yang Z, Xue C, Gong J, Sun S, et al. SBI0206965, a novel inhibitor of Ulk1, suppresses non-small cell lung cancer cell growth by modulating both autophagy and apoptosis pathways. *Oncol Rep* (2017) 37 (6):3449–58. doi: 10.3892/or.2017.5635
28. Qiu L, Zhou G, Cao S. Targeted inhibition of ULK1 enhances daunorubicin sensitivity in acute myeloid leukemia. *Life Sci* (2020) 243:117234. doi: 10.1016/j.lfs.2019.117234
29. Liu J, Xia H, Kim M, Xu L, Li Y, Zhang L, et al. Beclin1 controls the levels of p53 by regulating the deubiquitination activity of USP10 and USP13. *Cell* (2011) 147(1):223–34. doi: 10.1016/j.cell.2011.08.037
30. Bryant KL, Stalnecker CA, Zeitouni D, Klomp JE, Peng S, Tikunov AP, et al. Combination of ERK and autophagy inhibition as a treatment approach for pancreatic cancer. *Nat Med* (2019) 25(4):628–40. doi: 10.1038/s41591-019-0368-8

31. Chen Y, Xie X, Wang C, Hu Y, Zhang H, Zhang L, et al. Dual targeting of NUAK1 and ULK1 using the multitargeted inhibitor MRT68921 exerts potent antitumor activities. *Cell Death Dis* (2020) 11(8):712. doi: 10.1038/s41419-020-02885-0
32. Chaachouay H, Ohneseit P, Toulany M, Kehlbach R, Multhoff G, Rodemann HP. Autophagy contributes to resistance of tumor cells to ionizing radiation. *Radiother Oncol* (2011) 99(3):287–92. doi: 10.1016/j.radonc.2011.06.002
33. Chen Y, Li X, Guo L, Wu X, He C, Zhang S, et al. Combining radiation with autophagy inhibition enhances suppression of tumor growth and angiogenesis in esophageal cancer. *Mol Med Rep* (2015) 12(2):1645–52. doi: 10.3892/mmr.2015.3623
34. Pan B, Chen D, Huang J, Wang R, Feng B, Song H, et al. HMGB1-mediated autophagy promotes docetaxel resistance in human lung adenocarcinoma. *Mol Cancer* (2014) 13:165. doi: 10.1186/1476-4598-13-165
35. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* (2008) 359(4):378–90. doi: 10.1056/NEJMoa0708857
36. Prieto-Dominguez N, Ordonez R, Fernandez A, Garcia-Palomo A, Muntane J, Gonzalez-Gallego J, et al. Modulation of Autophagy by Sorafenib: Effects on Treatment Response. *Front Pharmacol* (2016) 7:151. doi: 10.3389/fphar.2016.00151
37. Yuan H, Li AJ, Ma SL, Cui LJ, Wu B, Yin L, et al. Inhibition of autophagy signi fi cantly enhances combination therapy with sorafenib and HDAC inhibitors for human hepatoma cells. *World J Gastroenterol* (2014) 20 (17):4953–62. doi: 10.3748/wjg.v20.i17.4953
38. Wu W, Ma J, Shao N, Shi Y, Liu R, Li W, et al. Co-Targeting IGF-1R and Autophagy Enhances the Effects of Cell Growth Suppression and Apoptosis Induced by the IGF-1R Inhibitor NVP-AEW541 in Triple-Negative Breast Cancer Cells. *PloS One* (2017) 12(1):e0169229. doi: 10.1371/journal.pone.0169229
39. Xu H, Yu Y, Marciniak D, Rishi AK, Sarkar FH, Kucuk O, et al. Epidermal growth factor receptor (EGFR)-related protein inhibits multiple members of the EGFR family in colon and breast cancer cells. *Mol Cancer Ther* (2005) 4 (3):435–42. doi: 10.1158/1535-7163.MCT-04-0280
40. Liu Z, He K, Ma Q, Yu Q, Liu C, Ndege I, et al. Autophagy inhibitor facilitates gefitinib sensitivity in vitro and in vivo by activating mitochondrial apoptosis in triple negative breast cancer. *PloS One* (2017) 12(5):e0177694. doi: 10.1371/journal.pone.0177694
41. Benjamin D, Colombi M, Moroni C, Hall MN. Rapamycin passes the torch: a new generation of mTOR inhibitors. *Nat Rev Drug Discovery* (2011) 10 (11):868–80. doi: 10.1038/nrd3531
42. Kim TS. Hope as a mode of coping in amyotrophic lateral sclerosis. *J Neurosci Nurs* (1989) 21(6):342–7. doi: 10.1097/01376517-198912000-00003
43. Dyczynski M, Yu Y, Otrocka M, Parpal S, Braga T, Henley AB, et al. Targeting autophagy by small molecule inhibitors of vacuolar protein sorting 34 (Vps34) improves the sensitivity of breast cancer cells to Sunitinib. *Cancer Lett* (2018) 435:32–43. doi: 10.1016/j.canlet.2018.07.028
44. Noman MZ, Parpal S, Van Moer K, Xiao M, Yu Y, Viklund J, et al. Inhibition of Vps34 reprograms cold into hot inflamed tumors and improves anti-PD-1/PD-L1 immunotherapy. *Sci Adv* (2020) 6(18):eaax7881. doi: 10.1126/sciadv.aax7881
45. Park JM, Tougeron D, Huang S, Okamoto K, Sinicrope FA. Beclin 1 and UVRAG confer protection from radiation-induced DNA damage and maintain centrosome stability in colorectal cancer cells. *PloS One* (2014) 9 (6):e100819. doi: 10.1371/journal.pone.0100819
46. Xie FJ, Zheng QQ, Qin J, Zhang LL, Han N, Mao WM. Autophagy Inhibition Stimulates Apoptosis in Oesophageal Squamous Cell Carcinoma Treated with Fasudil. *J Cancer* (2018) 9(6):1050–6. doi: 10.7150/jca.23388
47. Wu CL, Liu JF, Liu Y, Wang YX, Fu KF, Yu XJ, et al. Beclin1 inhibition enhances paclitaxelmediated cytotoxicity in breast cancer in vitro and in vivo. *Int J Mol Med* (2019) 43(4):1866–78. doi: 10.3892/ijmm.2019.4089
48. Xi G, Hu X, Wu B, Jiang H, Young CY, Pang Y, et al. Autophagy inhibition promotes paclitaxel-induced apoptosis in cancer cells. *Cancer Lett* (2011) 307(2):141–8. doi: 10.1016/j.canlet.2011.03.026
49. Zhang Q, Si S, Schoen S, Chen J, Jin XB, Wu G. Suppression of autophagy enhances preferential toxicity of paclitaxel to folliculin-deficient renal cancer cells. *J Exp Clin Cancer Res* (2013) 32:99. doi: 10.1186/1756-9966-32-99
50. Liu S, Li X. Autophagy inhibition enhances sensitivity of endometrial carcinoma cells to paclitaxel. *Int J Oncol* (2015) 46(6):2399–408. doi: 10.3892/ijo.2015.2937
51. Zhang SF, Wang XY, Fu ZQ, Peng QH, Zhang JY, Ye F, et al. TXNDC17 promotes paclitaxel resistance via inducing autophagy in ovarian cancer. *Autophagy* (2015) 11(2):225–38. doi: 10.1080/15548627.2014.998931
52. Song Y, Li W, Peng X, Xie J, Li H, Tan G. Inhibition of autophagy results in a reversal of taxol resistance in nasopharyngeal carcinoma by enhancing taxol-induced caspase-dependent apoptosis. *Am J Transl Res* (2017) 9(4):1934–42. doi: 10.1097/cad.0b013e3283363980
53. Zhen Z, Yang K, Ye L, You Z, Chen R, Liu Y, et al. Suberoylanilide hydroxamic acid sensitizes neuroblastoma to paclitaxel by inhibiting thioredoxin-related protein 14-mediated autophagy. *Cancer Sci* (2017) 108 (7):1485–92. doi: 10.1111/cas.13279
54. Shao S, Li S, Qin Y, Wang X, Yang Y, Bai H, et al. Spautin-1, a novel autophagy inhibitor, enhances imatinib-induced apoptosis in chronic myeloid leukemia. *Int J Oncol* (2014) 44(5):1661–8. doi: 10.3892/ijo.2014.2313
55. Yu Y, Yang L, Zhao M, Zhu S, Kang R, Vernon P, et al. Targeting microRNA-30a-mediated autophagy enhances imatinib activity against human chronic myeloid leukemia cells. *Leukemia* (2012) 26(8):1752–60. doi: 10.1038/leu.2012.65
56. Clark GM, Osborne CK, McGuire WL. Correlations between estrogen receptor, progesterone receptor, and patient characteristics in human breast cancer. *J Clin Oncol* (1984) 2(10):1102–9. doi: 10.1200/JCO.1984.2.10.1102
57. Gu Y, Chen T, Li G, Xu C, Xu Z, Zhang J, et al. Lower Beclin 1 downregulates HER2 expression to enhance tamoxifen sensitivity and predicts a favorable outcome for ER positive breast cancer. *Oncotarget* (2017) 8(32):52156–77. doi: 10.18632/oncotarget.11044
58. Zhang Y, Cheng Y, Ren X, Zhang L, Yap KL, Wu H, et al. NAC1 modulates sensitivity of ovarian cancer cells to cisplatin by altering the HMGB1-mediated autophagic response. *Oncogene* (2012) 31(8):1055–64. doi: 10.1038/onc.2011.290
59. Ma B, Liang LZ, Liao GQ, Liang YJ, Liu HC, Zheng GS, et al. Inhibition of autophagy enhances cisplatin cytotoxicity in human adenoid cystic carcinoma cells of salivary glands. *J Oral Pathol Med* (2013) 42(10):774–80. doi: 10.1111/jop.12066
60. Zhang R, Wang R, Chen Q, Chang H. Inhibition of autophagy using 3-methyladenine increases cisplatin-induced apoptosis by increasing endoplasmic reticulum stress in U251 human glioma cells. *Mol Med Rep* (2015) 12(2):1727–32. doi: 10.3892/mmr.2015.3588
61. Schlutermann D, Skowron MA, Berleth N, Bohler P, Deitersen J, Stuhldreier F, et al. Targeting urothelial carcinoma cells by combining cisplatin with a specific inhibitor of the autophagy-inducing class III PtdIns3K complex. *Urol Oncol* (2018) 36(4):160 e161–160 e113. doi: 10.1016/j.urolonc.2017.11.021
62. Giampieri R, Caporale M, Pietrantonio F, De Braud F, Negri FV, Giuliani F, et al. Second-line angiogenesis inhibition in metastatic colorectal cancer patients: Straightforward or overcrowded? *Crit Rev Oncol Hematol* (2016) 100:99–106. doi: 10.1016/j.critrevonc.2016.02.005
63. Stratigos M, Matikas A, Voutsina A, Mavroudis D, Georgoulias V. Targeting angiogenesis in small cell lung cancer. *Transl Lung Cancer Res* (2016) 5 (4):389–400. doi: 10.21037/tlcr.2016.08.04
64. Jain RK, Gandhi S, George S. Second-line systemic therapy in metastatic renal-cell carcinoma: A review. *Urol Oncol* (2017) 35(11):640–6. doi: 10.1016/j.urolonc.2017.08.010
65. Zhao Z, Xia G, Li N, Su R, Chen X, Zhong L. Autophagy Inhibition Promotes Bevacizumab-induced Apoptosis and Proliferation Inhibition in Colorectal Cancer Cells. *J Cancer* (2018) 9(18):3407–16. doi: 10.7150/jca.24201
66. Peeters M, Price T. Biologic therapies in the metastatic colorectal cancer treatment continuum–applying current evidence to clinical practice. *Cancer Treat Rev* (2012) 38(5):397–406. doi: 10.1016/j.ctrv.2011.08.002
67. Mele L, Del Vecchio V, Liccardo D, Prisco C, Schwerdtfeger M, Robinson N, et al. The role of autophagy in resistance to targeted therapies. *Cancer Treat Rev* (2020) 88:102043. doi: 10.1016/j.ctrv.2020.102043
68. Baginska J, Viry E, Berchem G, Poli A, Noman MZ, van Moer K, et al. Granzyme B degradation by autophagy decreases tumor cell susceptibility to

natural killer-mediated lysis under hypoxia. *Proc Natl Acad Sci USA* (2013) 110(43):17450–5. doi: 10.1073/pnas.1304790110
69. Mgrditchian T, Arakelian T, Paggetti J, Noman MZ, Viry E, Moussay E, et al. Targeting autophagy inhibits melanoma growth by enhancing NK cells infiltration in a CCL5-dependent manner. *Proc Natl Acad Sci USA* (2017) 114(44):E9271–9. doi: 10.1073/pnas.1703921114
70. Rosenfeldt MT, O'Prey J, Morton JP, Nixon C, MacKay G, Mrowinska A, et al. p53 status determines the role of autophagy in pancreatic tumour development. *Nature* (2013) 504(7479):296–300. doi: 10.1038/nature12865
71. Maes H, Kuchnio A, Peric A, Moens S, Nys K, De Bock K, et al. Tumor vessel normalization by chloroquine independent of autophagy. *Cancer Cell* (2014) 26(2):190–206. doi: 10.1016/j.ccr.2014.06.025
72. Rao S, Tortola L, Perlot T, Wirnsberger G, Novatchkova M, Nitsch R, et al. A dual role for autophagy in a murine model of lung cancer. *Nat Commun* (2014) 5:3056. doi: 10.1038/ncomms4056
73. Poillet-Perez L, Xie X, Zhan L, Yang Y, Sharp DW, Hu ZS, et al. Autophagy maintains tumour growth through circulating arginine. *Nature* (2018) 563 (7732):569–73. doi: 10.1038/s41586-018-0697-7
74. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* (2016) 12(1):1–222. doi: 10.1080/15548627.2015.1100356
75. Qian Z, Lin C, Espinosa R, LeBeau M, Rosner MR. Cloning and characterization of MST4, a novel Ste20-like kinase. *J Biol Chem* (2001) 276(25):22439–45. doi: 10.1074/jbc.M009323200
76. Li T, Deng L, He X, Jiang G, Hu F, Ye S, et al. MST4 Predicts Poor Prognosis And Promotes Metastasis By Facilitating Epithelial-Mesenchymal Transition In Gastric Cancer. *Cancer Manag Res* (2019) 11:9353–69. doi: 10.2147/CMAR.S219689
77. Madsen CD, Hooper S, Tozluoglu M, Bruckbauer A, Fletcher G, Erler JT, et al. STRIPAK components determine mode of cancer cell migration and metastasis. *Nat Cell Biol* (2015) 17(1):68–80. doi: 10.1038/ncb3083
78. Thompson BJ, Sahai E. MST kinases in development and disease. *J Cell Biol* (2015) 210(6):871–82. doi: 10.1083/jcb.201507005
79. Huang T, Kim CK, Alvarez AA, Pangeni RP, Wan X, Song X, et al. MST4 Phosphorylation of ATG4B Regulates Autophagic Activity, Tumorigenicity, and Radioresistance in Glioblastoma. *Cancer Cell* (2017) 32840-855(6):e848. doi: 10.1016/j.ccell.2017.11.005
80. Akin D, Wang SK, Habibzadegah-Tari P, Law B, Ostrov D, Li M, et al. A novel ATG4B antagonist inhibits autophagy and has a negative impact on osteosarcoma tumors. *Autophagy* (2014) 10(11):2021–35. doi: 10.4161/auto.32229
81. Kurdi A, Cleenewerck M, Vangestel C, Lyssens S, Declercq W, Timmermans JP, et al. ATG4B inhibitors with a benzotropolone core structure block autophagy and augment efficiency of chemotherapy in mice. *Biochem Pharmacol* (2017) 138:150–62. doi: 10.1016/j.bcp.2017.06.119
82. Chu J, Fu Y, Xu J, Zheng X, Gu Q, Luo X, et al. ATG4B inhibitor FMK-9a induces autophagy independent on its enzyme inhibition. *Arch Biochem Biophys* (2018) 644:29–36. doi: 10.1016/j.abb.2018.03.001
83. Fu Y, Hong L, Xu J, Zhong G, Gu Q, Gu Q, et al. Discovery of a small molecule targeting autophagy via ATG4B inhibition and cell death of colorectal cancer cells in vitro and in vivo. *Autophagy* (2019) 15(2):295–311. doi: 10.1080/15548627.2018.1517073
84. Liu PF, Tsai KL, Hsu CJ, Tsai WL, Cheng JS, Chang HW, et al. Drug Repurposing Screening Identifies Tioconazole as an ATG4 Inhibitor that Suppresses Autophagy and Sensitizes Cancer Cells to Chemotherapy. *Theranostics* (2018) 8(3):830–45. doi: 10.7150/thno.22012
85. Bortnik S, Choutka C, Horlings HM, Leung S, Baker JH, Lebovitz C, et al. Identification of breast cancer cell subtypes sensitive to ATG4B inhibition. *Oncotarget* (2016) 7(41):66970–88. doi: 10.18632/oncotarget.11408
86. Chen J, Zhang L, Zhou H, Wang W, Luo Y, Yang H, et al. Inhibition of autophagy promotes cisplatin-induced apoptotic cell death through Atg5 and Beclin 1 in A549 human lung cancer cells. *Mol Med Rep* (2018) 17 (5):6859–65. doi: 10.3892/mmr.2018.8686
87. Chittaranjan S, Bortnik S, Dragowska WH, Xu J, Abeysundara N, Leung A, et al. Autophagy inhibition augments the anticancer effects of epirubicin treatment in anthracycline-sensitive and -resistant triple-negative breast cancer. *Clin Cancer Res* (2014) 20(12):3159–73. doi: 10.1158/1078-0432.CCR-13-2060
88. O'Donovan TR, O'Sullivan GC, McKenna SL. Induction of autophagy by drug-resistant esophageal cancer cells promotes their survival and recovery following treatment with chemotherapeutics. *Autophagy* (2011) 7(5):509–24. doi: 10.4161/auto.7.6.15066
89. Zheng B, Zhu H, Gu D, Pan X, Qian L, Xue B, et al. MiRNA-30a-mediated autophagy inhibition sensitizes renal cell carcinoma cells to sorafenib. *Biochem Biophys Res Commun* (2015) 459(2):234–9. doi: 10.1016/j.bbrc.2015.02.084
90. Yu L, Chen Y, Tooze SA. Autophagy pathway: Cellular and molecular mechanisms. *Autophagy* (2018) 14(2):207–15. doi: 10.1080/15548627.2017.1378838
91. Chen D, Xie J, Fiskesund R, Dong W, Liang X, Lv J, et al. Chloroquine modulates antitumor immune response by resetting tumor-associated macrophages toward M1 phenotype. *Nat Commun* (2018) 9(1):873. doi: 10.1038/s41467-018-03225-9
92. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell* (2008) 132(1):27–42. doi: 10.1016/j.cell.2007.12.018
93. Itakura E, Kishi-Itakura C, Mizushima N. The hairpin-type tail-anchored SNARE syntaxin 17 targets to autophagosomes for fusion with endosomes/lysosomes. *Cell* (2012) 151(6):1256–69. doi: 10.1016/j.cell.2012.11.001
94. Zhou F, Yang X, Zhao H, Liu Y, Feng Y, An R, et al. Down-regulation of OGT promotes cisplatin resistance by inducing autophagy in ovarian cancer. *Theranostics* (2018) 8(19):5200–12. doi: 10.7150/thno.27806
95. Chen Y, Meng D, Wang H, Sun R, Wang D, Wang S, et al. VAMP8 facilitates cellular proliferation and temozolomide resistance in human glioma cells. *Neuro Oncol* (2015) 17(3):407–18. doi: 10.1093/neuonc/nou219
96. Fu R, Deng Q, Zhang H, Hu X, Li Y, Liu Y, et al. A novel autophagy inhibitor berbamine blocks SNARE-mediated autophagosome-lysosome fusion through upregulation of BNIP3. *Cell Death Dis* (2018) 9(2):243. doi: 10.1038/s41419-018-0276-8
97. Mou L, Liang B, Liu G, Jiang J, Liu J, Zhou B, et al. Berbamine exerts anticancer effects on human colon cancer cells via induction of autophagy and apoptosis, inhibition of cell migration and MEK/ERK signalling pathway. *J BUON* (2019) 24(5):1870–5.
98. Zhang H, Jiao Y, Shi C, Song X, Chang Y, Ren Y, et al. Berbamine suppresses cell proliferation and promotes apoptosis in ovarian cancer partially via the inhibition of Wnt/beta-catenin signaling. *Acta Biochim Biophys Sin (Shanghai)* (2018) 50(6):532–9. doi: 10.1093/abbs/gmy036
99. Morell C, Bort A, Vara-Ciruelos D, Ramos-Torres A, Altamirano-Dimas M, Diaz-Laviada I, et al. Up-Regulated Expression of LAMP2 and Autophagy Activity during Neuroendocrine Differentiation of Prostate Cancer LNCaP Cells. *PloS One* (2016) 11(9):e0162977. doi: 10.1371/journal.pone.0162977
100. Jamali L, Moradi A, Ganji M, Ayati M, Kazeminezhad B, Fazeli Attar Z, et al. Potential Prognostic Role for SPOP, DAXX, RARRES1, and LAMP2 as an Autophagy Related Genes in Prostate Cancer. *Urol J* (2020) 17(2):156–63. doi: 10.22037/uj.v0i0.4935
101. Huang CF, Deng WW, Zhang L, Zhang WF, Sun ZJ. Expression of LC3, LAMP2, KEAP1 and NRF2 in Salivary Adenoid Cystic Carcinoma. *Pathol Oncol Res* (2016) 22(1):109–14. doi: 10.1007/s12253-015-9981-0
102. Koukourakis MI, Kalamida D, Mitrakas A, Pouliliou S, Kalamida S, Sivridis E, et al. Intensified autophagy compromises the efficacy of radiotherapy against prostate cancer. *Biochem Biophys Res Commun* (2015) 461(2):268–74. doi: 10.1016/j.bbrc.2015.04.014
103. Safaei R, Larson BJ, Cheng TC, Gibson MA, Otani S, Naerdemann W, et al. Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells. *Mol Cancer Ther* (2005) 4 (10):1595–604. doi: 10.1158/1535-7163.MCT-05-0102
104. Robert G, Auberger P. Azacitidine resistance caused by LAMP2 deficiency: a therapeutic window for the use of autophagy inhibitors in MDS/AML patients? *Autophagy* (2019) 15(5):927–9. doi: 10.1080/15548627.2019.1586259
105. Kuchitsu Y, Fukuda M. Revisiting Rab7 Functions in Mammalian Autophagy: Rab7 Knockout Studies. *Cells* (2018) 7(11):215. doi: 10.3390/cells7110215
106. Liu H, Xu J, Yao Q, Zhang Z, Guo Q, Lin J. Rab7 Is Associated with Poor Prognosis of Gastric Cancer and Promotes Proliferation, Invasion, and Migration of Gastric Cancer Cells. *Med Sci Monit* (2020) 26:e922217. doi: 10.12659/MSM.922217

107. Guerra F, Bucci C. Role of the RAB7 Protein in Tumor Progression and Cisplatin Chemoresistance. *Cancers (Basel)* (2019) 11(8):1096. doi: 10.3390/cancers11081096
108. Guerra F, Paiano A, Migoni D, Girolimetti G, Perrone AM, De Iaco P, et al. Modulation of RAB7A Protein Expression Determines Resistance to Cisplatin through Late Endocytic Pathway Impairment and Extracellular Vesicular Secretion. *Cancers (Basel)* (2019) 11(1):52. doi: 10.3390/cancers11010052
109. Bhargava HK, Tabata K, Byck JM, Hamasaki M, Farrell DP, Anishchenko I, et al. Structural basis for autophagy inhibition by the human Rubicon-Rab7 complex. *Proc Natl Acad Sci USA* (2020) 117(29):17003–10. doi: 10.1073/pnas.2008030117
110. Mauthe M, Orhon I, Rocchi C, Zhou X, Luhr M, Hijlkema KJ, et al. Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. *Autophagy* (2018) 14(8):1435–55. doi: 10.1080/15548627.2018.1474314
111. Tsubone T, Rocha C, Tonolli P, II-Sei W, Stolf B. In vitro Autophagy Modulation with Chloroquine: Some Lessons to Learn. *Adv Biochem Biotechnol* (2020) 5:1098. doi: 10.29011/2574-7258.001098
112. Wang F, Tang J, Li P, Si S, Yu H, Yang X, et al. Chloroquine Enhances the Radiosensitivity of Bladder Cancer Cells by Inhibiting Autophagy and Activating Apoptosis. *Cell Physiol Biochem* (2018) 45(1):54–66. doi: 10.1159/000486222
113. Ye H, Chen M, Cao F, Huang H, Zhan R, Zheng X. Chloroquine, an autophagy inhibitor, potentiates the radiosensitivity of glioma initiating cells by inhibiting autophagy and activating apoptosis. *BMC Neurol* (2016) 16(1):178. doi: 10.1186/s12883-016-0700-6
114. Roy LO, Poirier MB, Fortin D. Chloroquine inhibits the malignant phenotype of glioblastoma partially by suppressing TGF-beta. *Invest New Drugs* (2015) 33(5):1020–31. doi: 10.1007/s10637-015-0275-x
115. Ratikan JA, Sayre JW, Schaue D. Chloroquine engages the immune system to eradicate irradiated breast tumors in mice. *Int J Radiat Oncol Biol Phys* (2013) 87(4):761–8. doi: 10.1016/j.ijrobp.2013.07.024
116. Yoon YH, Cho KS, Hwang JJ, Lee SJ, Choi JA, Koh JY. Induction of lysosomal dilatation, arrested autophagy, and cell death by chloroquine in cultured ARPE-19 cells. *Invest Ophthalmol Vis Sci* (2010) 51(11):6030–7. doi: 10.1167/iovs.10-5278
117. Maycotte P, Aryal S, Cummings CT, Thorburn J, Morgan MJ, Thorburn A. Chloroquine sensitizes breast cancer cells to chemotherapy independent of autophagy. *Autophagy* (2012) 8(2):200–12. doi: 10.4161/auto.8.2.18554
118. Schonewolf CA, Mehta M, Schiff D, Wu H, Haffty BG, Karantza V, et al. Autophagy inhibition by chloroquine sensitizes HT-29 colorectal cancer cells to concurrent chemoradiation. *World J Gastrointest Oncol* (2014) 6(3):74–82. doi: 10.4251/wjgo.v6.i3.74
119. Geng Y, Kohli L, Klocke BJ, Roth KA. Chloroquine-induced autophagic vacuole accumulation and cell death in glioma cells is p53 independent. *Neuro Oncol* (2010) 12(5):473–81. doi: 10.1093/neuonc/nop048
120. Eng CH, Wang Z, Tkach D, Toral-Barza L, Ugwonali S, Liu S, et al. Macroautophagy is dispensable for growth of KRAS mutant tumors and chloroquine efficacy. *Proc Natl Acad Sci USA* (2016) 113(1):182–7. doi: 10.1073/pnas.1515617113
121. Lin JF, Lin YC, Tsai TF, Chen HE, Chou KY, Hwang TI. Cisplatin induces protective autophagy through activation of BECN1 in human bladder cancer cells. *Drug Des Devel Ther* (2017) 11:1517–33. doi: 10.2147/DDDT.S126464
122. Aga T, Endo K, Tsuji A, Aga M, Moriyama-Kita M, Ueno T, et al. Inhibition of autophagy by chloroquine makes chemotherapy in nasopharyngeal carcinoma more efficient. *Auris Nasus Larynx* (2019) 46(3):443–50. doi: 10.1016/j.anl.2018.10.013
123. Zhao XG, Sun RJ, Yang XY, Liu DY, Lei DP, Jin T, et al. Chloroquine-enhanced efficacy of cisplatin in the treatment of hypopharyngeal carcinoma in xenograft mice. *PloS One* (2015) 10(4):e0126147. doi: 10.1371/journal.pone.0126147
124. Choi HS, Jeong EH, Lee TG, Kim SY, Kim HR, Kim CH. Autophagy Inhibition with Monensin Enhances Cell Cycle Arrest and Apoptosis Induced by mTOR or Epidermal Growth Factor Receptor Inhibitors in Lung Cancer Cells. *Tuberc Respir Dis (Seoul)* (2013) 75(1):9–17. doi: 10.4046/trd.2013.75.1.9
125. Ketola K, Vainio P, Fey V, Kallioniemi O, Iljin K. Monensin is a potent inducer of oxidative stress and inhibitor of androgen signaling leading to apoptosis in prostate cancer cells. *Mol Cancer Ther* (2010) 9(12):3175–85. doi: 10.1158/1535-7163.MCT-10-0368
126. Wiedmer T, Blank A, Pantasis S, Normand L, Bill R, Krebs P, et al. Autophagy Inhibition Improves Sunitinib Efficacy in Pancreatic Neuroendocrine Tumors via a Lysosome-dependent Mechanism. *Mol Cancer Ther* (2017) 16(11):2502–15. doi: 10.1158/1535-7163.MCT-17-0136
127. Cufi S, Vazquez-Martin A, Oliveras-Ferraros C, Corominas-Faja B, Cuyas E, Lopez-Bonet E, et al. The anti-malarial chloroquine overcomes primary resistance and restores sensitivity to trastuzumab in HER2-positive breast cancer. *Sci Rep* (2013) 3:1–13. 2469. doi: 10.1038/srep02469
128. Zamame Ramirez JA, Romagnoli GG, Falasco BF, Gorgulho CM, Sanzochi Fogolin C, Dos Santos DC, et al. Blocking drug-induced autophagy with chloroquine in HCT-116 colon cancer cells enhances DC maturation and T cell responses induced by tumor cell lysate. *Int Immunopharmacol* (2020) 84:106495. doi: 10.1016/j.intimp.2020.106495
129. Yamamoto K, Venida A, Yano J, Biancur DE, Kakiuchi M, Gupta S, et al. Autophagy promotes immune evasion of pancreatic cancer by degrading MHC-I. *Nature* (2020) 581(7806):100–5. doi: 10.1038/s41586-020-2229-5
130. Chu HY, Wang W, Chen X, Jiang YE, Cheng R, Qi X, et al. Bafilomycin A1 increases the sensitivity of tongue squamous cell carcinoma cells to cisplatin by inhibiting the lysosomal uptake of platinum ions but not autophagy. *Cancer Lett* (2018) 423:105–12. doi: 10.1016/j.canlet.2018.03.003
131. Li LQ, Xie WJ, Pan D, Chen H, Zhang L. Inhibition of autophagy by bafilomycin A1 promotes chemosensitivity of gastric cancer cells. *Tumour Biol* (2016) 37(1):653–9. doi: 10.1007/s13277-015-3842-z
132. Fu W, Li X, Lu X, Zhang L, Li R, Zhang N, et al. A novel acridine derivative, LS-1-10 inhibits autophagic degradation and triggers apoptosis in colon cancer cells. *Cell Death Dis* (2017) 8(10):e3086. doi: 10.1038/cddis.2017.498
133. Takanezawa Y, Nakamura R, Kojima Y, Sone Y, Uraguchi S, Kiyono M. Cytochalasin E increased the sensitivity of human lung cancer A549cells to bortezomib via inhibition of autophagy. *Biochem Biophys Res Commun* (2018) 498(3):603–8. doi: 10.1016/j.bbrc.2018.03.029
134. Alizadeh J, Zeki AA, Mirzaei N, Tewary S, Rezaei Moghadam A, Glogowska A, et al. Mevalonate Cascade Inhibition by Simvastatin Induces the Intrinsic Apoptosis Pathway via Depletion of Isoprenoids in Tumor Cells. *Sci Rep* (2017) 7:44841. doi: 10.1038/srep44841
135. Shojaei S, Koleini N, Samiei E, Aghaei M, Cole LK, Alizadeh J, et al. Simvastatin increases temozolomide-induced cell death by targeting the fusion of autophagosomes and lysosomes. *FEBS J* (2020) 287(5):1005–34. doi: 10.1111/febs.15069
136. Kou X, Yang Y, Jiang X, Liu H, Sun F, Wang X, et al. Vorinostat and Simvastatin have synergistic effects on triple-negative breast cancer cells via abrogating Rab7 prenylation. *Eur J Pharmacol* (2017) 813:161–71. doi: 10.1016/j.ejphar.2017.08.022
137. Daskalaki I, Gkikas I, Tavernarakis N. Hypoxia and Selective Autophagy in Cancer Development and Therapy. *Front Cell Dev Biol* (2018) 6:104. doi: 10.3389/fcell.2018.00104
138. Carew JS, Espitia CM, Zhao W, Han Y, Visconte V, Phillips J, et al. Disruption of Autophagic Degradation with ROC-325 Antagonizes Renal Cell Carcinoma Pathogenesis. *Clin Cancer Res* (2017) 23(11):2869–79. doi: 10.1158/1078-0432.CCR-16-1742
139. Nawrocki ST, Han Y, Visconte V, Przychodzen B, Espitia CM, Phillips J, et al. The novel autophagy inhibitor ROC-325 augments the antileukemic activity of azacitidine. *Leukemia* (2019) 33(12):2971–4. doi: 10.1038/s41375-019-0529-2
140. Aldrich LN, Kuo SY, Castoreno AB, Goel G, Kuballa P, Rees MG, et al. Discovery of a Small-Molecule Probe for V-ATPase Function. *J Am Chem Soc* (2015) 137(16):5563–8. doi: 10.1021/jacs.5b02150
141. Carew JS, Espitia CM, Esquivel JA,2, Mahalingam D, Kelly KR, Reddy G, et al. Lucanthone is a novel inhibitor of autophagy that induces cathepsin D-mediated apoptosis. *J Biol Chem* (2011) 286(8):6602–13. doi: 10.1074/jbc.M110.151324
142. Martins WK, Costa ET, Cruz MC, Stolf BS, Miotto R, Cordeiro RM, et al. Parallel damage in mitochondrial and lysosomal compartments promotes efficient cell death with autophagy: The case of the pentacyclic triterpenoids. *Sci Rep* (2015) 5:12425. doi: 10.1038/srep12425
143. Martins WK, Gomide AB, Costa ET, Junqueira HC, Stolf BS, Itri R, et al. Membrane damage by betulinic acid provides insights into cellular aging.

Biochim Biophys Acta Gen Subj (2017) 1861(1 Pt A):3129–43. doi: 10.1016/j.bbagen.2016.10.018

144. Kang C, Elledge SJ. How autophagy both activates and inhibits cellular senescence. *Autophagy* (2016) 12(5):898–9. doi: 10.1080/15548627.2015.1121361
145. Cassidy LD, Young ARJ, Young CNJ, Soilleux EJ, Fielder E, Weigand BM, et al. Temporal inhibition of autophagy reveals segmental reversal of ageing with increased cancer risk. *Nat Commun* (2020) 11(1):307. doi: 10.1038/s41467-019-14187-x
146. Gewirtz DA. The Challenge of Developing Autophagy Inhibition as a Therapeutic Strategy. *Cancer Res* (2016) 76(19):5610–4. doi: 10.1158/0008-5472.CAN-16-0722

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Xiao, Benoit, Hasmim, Duhem, Vogin, Berchem, Noman and Janji. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Oncology

REVIEW
published: 26 February 2021
doi: 10.3389/fonc.2021.632956

Mechanobiology of Autophagy: The Unexplored Side of Cancer

Maria Paz Hernández-Cáceres[1], Leslie Munoz[2,3], Javiera M. Pradenas[4,5], Francisco Pena[2,3], Pablo Lagos[1], Pablo Aceiton[2,3], Gareth I. Owen[4,5,6], Eugenia Morselli[1,7], Alfredo Criollo[4,7,8], Andrea Ravasio[2] and Cristina Bertocchi[3]**

[1] Laboratory of Autophagy and Metabolism, Department of Physiology, Faculty of Biological Sciences, Pontificia Universidad Católica De Chile, Santiago, Chile, [2] Laboratory for Mechanobiology of Transforming Systems, Institute for Biological and Medical Engineering, Schools of Engineering, Medicine and Biological Sciences, Pontificia Universidad Católica de Chile, Santiago, Chile, [3] Laboratory for Molecular Mechanics of Cell Adhesion, Department of Physiology, Faculty of Biological Sciences, Pontificia Universidad Católica De Chile, Santiago, Chile, [4] Advanced Center for Chronic Diseases (ACCDiS), Santiago, Chile, [5] Laboratory of Investigation in Oncology, Faculty of Biological Sciences Pontificia Universidad Católica de Chile, Santiago, Chile, [6] Millennium Institute on Immunology and Immunotherapy, Santiago, Chile, [7] Autophagy Research Center, Santiago de Chile, Chile, [8] Facultad De Odontología, Instituto De Investigación En Ciencias Odontológicas (ICOD), Universidad De Chile, Santiago, Chile

OPEN ACCESS

Edited by:
Aamir Ahmad, University of Alabama at Birmingham, United States

Reviewed by:
Paola Costelli, University of Turin, Italy
Sujit Kumar Bhutia, National Institute of Technology Rourkela, India

***Correspondence:**
Cristina Bertocchi
cbertocchi@bio.puc.cl
Andrea Ravasio
andrea.ravasio@uc.cl

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *24 November 2020*
Accepted: *01 February 2021*
Published: *26 February 2021*

Citation:
Hernández-Cáceres MP, Munoz L, Pradenas JM, Pena F, Lagos P, Aceiton P, Owen GI, Morselli E, Criollo A, Ravasio A and Bertocchi C (2021) Mechanobiology of Autophagy: The Unexplored Side of Cancer. Front. Oncol. 11:632956. doi: 10.3389/fonc.2021.632956

Proper execution of cellular function, maintenance of cellular homeostasis and cell survival depend on functional integration of cellular processes and correct orchestration of cellular responses to stresses. Cancer transformation is a common negative consequence of mismanagement of coordinated response by the cell. In this scenario, by maintaining the balance among synthesis, degradation, and recycling of cytosolic components including proteins, lipids, and organelles the process of autophagy plays a central role. Several environmental stresses activate autophagy, among those hypoxia, DNA damage, inflammation, and metabolic challenges such as starvation. In addition to these chemical challenges, there is a requirement for cells to cope with mechanical stresses stemming from their microenvironment. Cells accomplish this task by activating an intrinsic mechanical response mediated by cytoskeleton active processes and through mechanosensitive protein complexes which interface the cells with their mechano-environment. Despite autophagy and cell mechanics being known to play crucial transforming roles during oncogenesis and malignant progression their interplay is largely overlooked. In this review, we highlight the role of physical forces in autophagy regulation and their potential implications in both physiological as well as pathological conditions. By taking a mechanical perspective, we wish to stimulate novel questions to further the investigation of the mechanical requirements of autophagy and appreciate the extent to which mechanical signals affect this process.

Keywords: autophagosome, biomembranes, cytoskeleton, mechanosensing, mechanotransduction

INTRODUCTION

At its completion in 2003, the Human Genome Project (1) was saluted as the tool to finally cure every cancer. Two decades later, this largely anticipated promise has yet to be delivered and the community of cancer researchers, which was once disproportionally focused on the central dogma of molecular biology, now embraces more holistic views. One of the most exciting frontiers of cancer research deals with understanding homeostatic processes during cancer development and how cancer cells respond to environmental stresses of a chemical and physical nature. In this context, the catabolic activity of autophagy is the key mechanism to maintain the balance between synthesis, degradation, and recycling of cytosolic components (2). These routine housekeeping functions provide a cellular mechanism to preserve homeostasis, enhance resilience to stresses and promote cell survival. Several environmental stresses activate autophagy, among those hypoxia, DNA damage, inflammation, and metabolic challenges such as starvation. Aside from challenges of chemical nature, cells are also exposed to stresses of mechanical nature, arising from environmental cues. Sensing of mechanical stress (mechanosensing) is mediated by force-induced conformational changes of mechanosensitive proteins directly or indirectly connected to the cytoskeleton and by mechanically activated ion channels (3, 4). Mechanosensing results in a modification of intracellular tension through reorganization of cytoskeletal and actomyosin contraction, which, in turn, integrate the mechanical signals into biochemical cascades (mechanotransduction), and, at longer time scale, lead to modification of gene expression (4). Hence, the physical properties of the microenvironment, such as extracellular matrix composition, stiffness, and architecture, have a profound impact on cellular genotype, phenotype, processes, tissue organization and overall biological function of the organism (4). This relation between mechanics and biological responses is also important during cancer transformation and progression, where the specific physical microenvironment of the tumor cells undergoes dramatic changes. These modifications of the tumor microniche are driven by enhanced cell contractility, increased pressure resulting from abnormal cell proliferation and growth of tumor mass, and alterations of composition, architecture and rheological properties of the surrounding extracellular matrix (5, 6). It has been reported that these mechanical changes correlate with activation of autophagy, which may be part of an integrated response to mechanical stresses employed by cancer cells to escape programmed cell death and to facilitate their adaptative response to the new mechanical environment (7). Furthermore, compelling evidence have suggested that autophagy impact several cancer hallmarks including cell motility and invasion, cancer stem cell viability and differentiation, epithelial to mesenchymal transition (EMT), resistance to apoptosis and anoikis, escape from immune surveillance and tumor cell dormancy (7, 8). However, the causative relation between cellular mechanics and autophagy and their interdependent role in cancer transformation are fragmentary and largely anecdotical. Here, we aim to review the autophagic process using a mechanical perspective and explore the crosstalk between mechanotransduction and cellular catabolism in order to access their possible contribution to cancer transformation and survival.

Abbreviations: AMPK, AMP-activated protein kinase; ATG, autophagy related protein; EGF, epidermal growth fctor; EMT, epithelial to mesenchymal transition; ER, endoplasmic reticulum; ESCRT, endosomal sorting complex required for transport; FAK, focal adhesion kinase; GABARAP, gamma-aminobutyric acid receptor-associated protein; JMY, junction-mediating and regulatory protein; LC3, microtubule-associated protein 1A/1B-light chain 3; LKB1, liver kinase B1; MLCK, myosin light-chain kinase; mTOR, mechanistic target of rapamycin; NPFs, nucleation-promoting factors; PE, phosphatidylethanolamine; PI(3)P, phosphatidylinositol 3-phosphate; PI3KC3: class III phosphatidylinositol 3-kinase; SNARE, soluble NSF attachment protein receptor; TAZ, transcriptional co-activator with PDZ-binding motif; TRP, transient receptor potential; VEFGR2, vascular endothelial growth factor receptor 2; VPS, vacuolar sorting protein; WASH, WAS protein family homolog; WHAMM, WASP homolog associated with actin, membranes and microtubules; YAP, Yes-associated protein; CAFs, cancer associated fibroblasts; ECM, extracellular matrix; FIP200, focal adhesion kinase family interacting protein of 200 kDa; ULK1, unc-51-like kinase 1.

ROLE OF CYTOSKELETON IN CELL MECHANICS

Vital functions of eukaryotic cells such as resistance to deformation, control of cellular shape, migration and transport of intracellular cargos depend on the activity of the cytoskeleton, an interconnected network of filamentous polymers, motor proteins and regulatory proteins (9). This network is composed by three interdependent structural components, namely microtubules, intermediate filaments, and microfilaments (actin) which are the engine of the cells as they convert chemical energy into mechanical energy *via* ATP-dependent polymerization and action of motor proteins. This mechanical energy is used to produce forces that displace cellular elements (e.g. formation of cellular protrusion, transport of cargos) and/or store elastic energy therein (e.g. cortical tension, cellular contractility). The whole process of autophagy being a sequence of membrane remodeling events is mechanically accomplished and coordinated by ATP-dependent cytoskeletal dynamics that lead to mechanical deformation and transport (10, 11). The cytoskeleton acts as an important framework for the modulation and control of correct positioning, tethering, docking, priming, fusion, and movement of organelles, such as autophagosomes and lysosomes. Actin cytoskeleton is composed by actin filaments and fibers whose assembly and disassembly generate web-like networks (Arp2/3-mediated branching) and bundles (formin-dependent crosslinking of filaments). These networks and bundles structurally support cellular membranes and determine their dynamics (12). Importantly, the action of molecular motors of the myosin family puts actin filaments under tension. Similar to a stretch coil, the release of this tension produces kinetic energy used for vesicle transport and membrane remodeling associated to autophagosome formation (13, 14). In addition, some myosins [i.e. myosin VI (15)] are directly involved in the transport of various cargos including autophagosomes (15). Furthermore, microtubules dynamics of polymerization and depolymerization and the action of

associated motor proteins [i.e. kinesin and dyneins (16, 17)] orchestrate the movement of pre-autophagosomal structures and autophagosomes across the cytoplasm during the process of autophagosome maturation (18, 19) and autolysosome bidirectional transport (20). The cooperation and competition between actin and microtubules are responsible for a large part of cellular mechanics. Together, these ATP-dependent cytoskeletal processes provide the mechanisms to overcome the energy barriers imposed by membrane elasticity and resistance to deformation that affect each step of the autophagic process (21). Finally, intermediate filaments (i.e. keratins and vimentin), which do not have evident dynamics and lack motor proteins, are thought to provide mechanical stability to the cell and its organelles (22). Intermediate filaments play a key role in autophagosome and lysosome positioning by providing a resistance to their free, unregulated movement (23). For instance, networks of vimentin cables have been observed to form cages around cellular organelles including the nucleus, endoplasmic reticulum, and mitochondria (24). Consistently with this regulatory function, pharmacological disruption of the vimentin network results in defective flow of the autophagic process (autophagic flux), the perinuclear position of autophagic vesicles and a loss of their region-specific localization at different stages of the process (23).

Step-By-Step Mechanics of Autophagy

From a mechanical point of view, the autophagic process can be divided into seven main stages, as depicted in **Figure 1**: initiation, nucleation, elongation, closure, autophagosome maturation and transportation toward the perinuclear region of the cell, fusion with the lysosome, and finally, cargo degradation and recycling (25).

Initiation Stage

Upon a chemical or mechanical stimulation the autophagic process begins, with the recruitment of core autophagy factors (**Figure 1**—initiation). This stage corresponds to the activation of the ULK1complex (26). As indicated in the schematic in

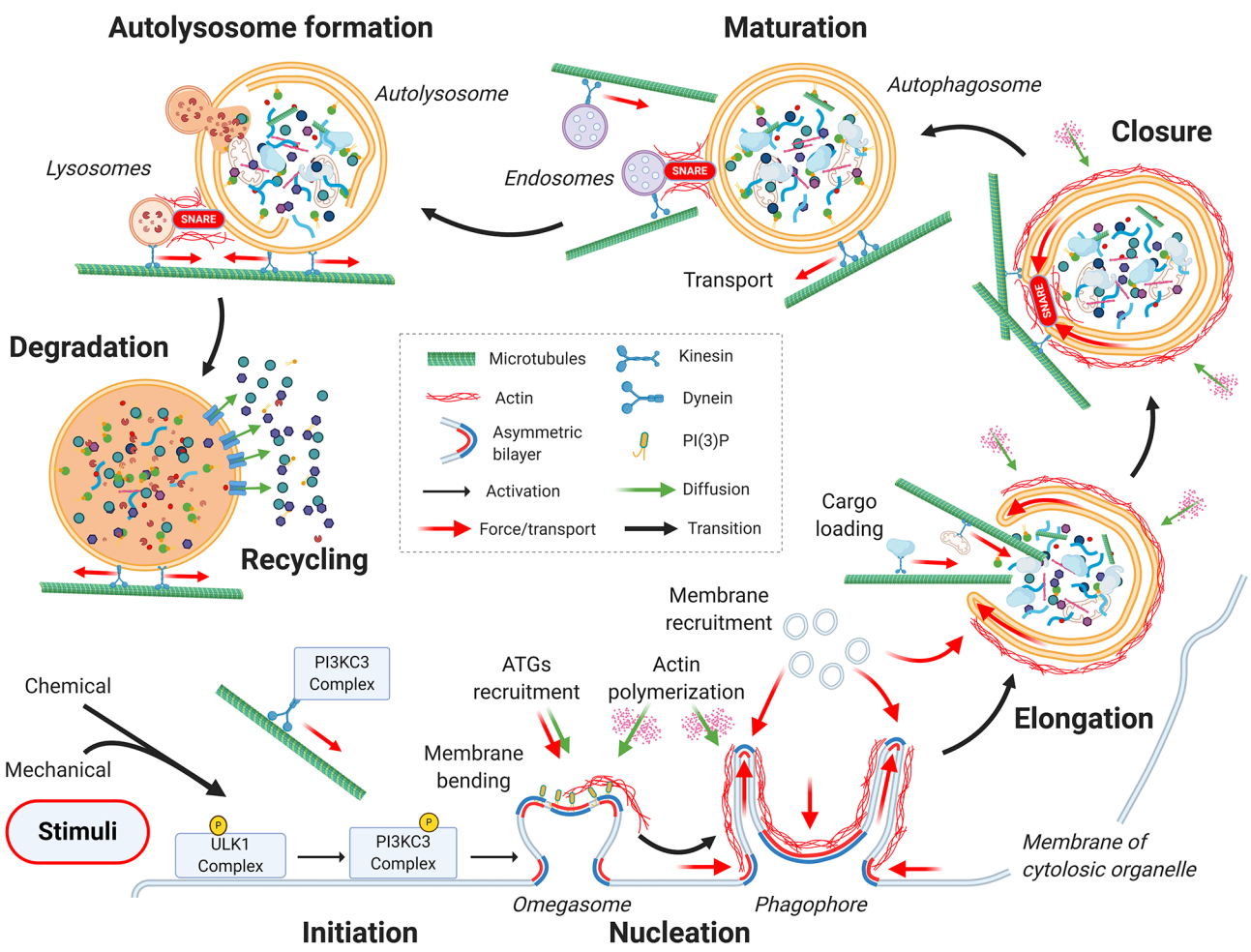


FIGURE 1 | Mechanics of the autophagic process. From a mechanical point of view, the autophagic process can be divided into seven main stages: initiation, nucleation, elongation, closure, autophagosome maturation, autolysosome formation, and finally, cargo degradation and recycling. Cytoskeletal active processes and membrane organization during the sequential steps of autophagy are highlighted. See the main text for details.

Figure 2, modulation of the ULK1 complex is achieved by enhancing the activity of AMPK (induced by ATP depletion) (27) and/or by inhibition of the mechanistic target of rapamycin complex 1 (mTORC1) which acts as repressor of autophagy and, under basal conditions, maintains ULK1 in an inactive conformation (27). Canonical initiation of autophagy entails that metabolic stresses (chemical stimuli), such as nutrient deprivation, cause mTORC1 dissociation from ULK1, which becomes active and binds to ATG13 and FIP200 (ULK1 complex—**Figure 2**). This early signaling triggers the downstream events of autophagosome formation (**Figure 1**—initiation). Whether mechanical stresses and signals may play a direct role in ULK1 activation is still unclear. It has been reported that mechanosensitive (that responds to mechanical stimuli) mTORC2 (28, 29) is in a negative feedback loop with mTORC1 (30, 31), thus could indirectly induce activation of ULK1 to initiate autophagy *via* inactivation of mTORC1-repressor function (32). Importantly, mTORC2 can be mechanically activated by mechanosensitive, focal adhesion kinase (FAK) (33) (**Figure 2**). In adherent cells, FAK is part of focal adhesion, a protein complex mediating cell/substrate adhesion. Decrease of mechanical forces at the focal adhesions, which may occur upon detachment of the cells from the substrate or due to changes in rheological properties of the extracellular matrix, induce FAK dissociation from the focal adhesion complex (34). Soluble FAK is free to phosphorylate (activate) mTORC2 and consequently initiate autophagy (33). Interestingly, mTORC2 can also activate AKT, which reestablishes the inhibitory activity of mTORC1 through an indirect signaling cascade (28). Hence, FAK, mTORC2 and AKT may provide a possible negative modulation or an off-switch to detain the autophagic process (**Figure 2**).

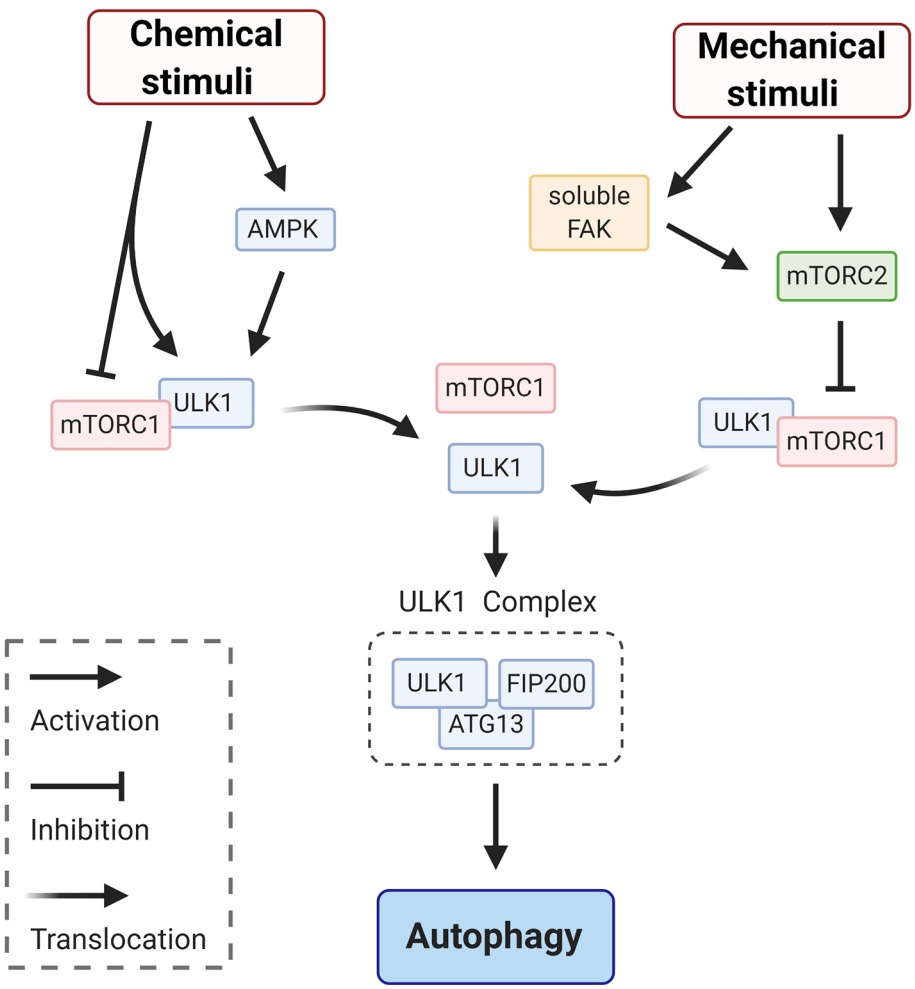


FIGURE 2 | Schematic representation of signaling circuits for ULK1 complex activation in autophagy initiation. Initiation of autophagy *via* ULK1 signaling entails mTORC1 dissociation from ULK1, which becomes active and binds to ATG13 and FIP200 to form ULK1 complex. This dissociation and the following signaling cascade can be elicited by chemical stimuli, *via* e.g. enhancing the activity of AMPK and/or by mechanical stimuli. This second pathway is achieved *via* inhibition of mTORC1 by the mechanosensitive mTORC2, which responds to various mechanical stimuli. For instance, mTORC2 can be directly activated by the soluble form of focal adhesion kinase (FAK) that is releases from focal adhesion at low traction forces (e.g. detachment of the cells from the substrate).

Nucleation

The process of formation of the initial complex of membranes that will elongate and mature into the autophagosome, begins with the binding of activated ULK1 complex to or in proximity of the sites for phagophore formation (35) (**Figure 1**—nucleation). In yeast, the phagophore assembly site is found in a dedicated and confined space between the endoplasmic reticulum (ER) exit site (36, 37) and the vacuole (yeast degradative lysosome) (38). Interestingly, in yeast, the autophagosome remains in this space throughout the autophagic process. Conversely, in mammalian cells, the phagophore assembly sites may be found at different cytoplasmic locations such as ER, ER-mitochondria junctions, or ER-plasma membrane, as well as specific subdomains of the plasma membrane containing the primary cilium (39–41). As consequence, the autophagosome needs to be transported throughout the cytosol for proper maturation. These differences between mammalian and yeast cells may reflect differences in structural complexity and spatial patterns of stress signal. It is tempting to speculate that the higher spatial complexity of the autophagic process seen in mammalian cells could be a result of higher mechanical complexity as compared to the wall-protected and sedentary yeast cells. The formation of the autophagosome begins with a curved membranous structure, named the omegasome for its shape resembling the Greek letter omega (42). The omegasome folds as a double membrane digit that receives lipids from most of the internal compartments of the cell (42, 43). The omegasome grows into a cup-shaped double membrane, known as phagophore or isolation membrane, which is typically connected with the ER membrane at its base (44). Eventually, the connection between the ER and the omegasome is sealed off and an independent double-membrane organelle is formed (42). To achieve this, several mechanical and energetic requirements need to be met. These include recruitment of specific ATGs, actin cytoskeleton to support and direct the curved membrane, and the recruitment of the necessary material, in particular phospholipids, to allow the *de novo* buildup of the phagophore (45) (see **Figure 1**—nucleation). ULK1 complex is responsible for the initiation all these mechanisms. As first step, ULK1 recruits and activates PI3KC3, a kinase complex formed by VPS34, Beclin-1, VPS15 and ATG14 (46). Activation of PI3KC3 occurs *via* ULK1 phosphorylation of Ambra1 (47), a Beclin-1 interacting protein. The PI3KC3 complex, which is tethered to the cytoskeleton through an interaction between the Ambra1 and dynein light chains (47), leads to PI3KC3 release from dynein light chain and the microtubule network, enabling the complex activation and translocation to the omegasome. In this location, PI3KC3 phosphorylates Phosphatidylinositol to generate Phosphatidylinositol 3-phosphate (PI(3)P), which promotes membrane bending and the recruitment of the additional ATG proteins required in the later stages of autophagosome formation (44) (see **Figure 1**—nucleation; ATGs recruitment). In addition to PI(3)P, membrane bending is also sustained by Atg17 (yeast counterpart of FIP200), a specific scaffolding protein that may also provide a curvature-sensing mechanism (48, 49). The Atg17 dimer has multiple hydrophobic residues that favor membrane interaction. Atg17 dimers arrange to tether the fused vesicles together, adopting a peculiar double-crescent shape (48) which is ideal to induce and sustain membrane bending. In addition, PI(3)P recruits specific membrane associated nucleation-promoting factors (NPFs), such as WHAMM (50), JMY (51), and WASH (52). In response to the specific localization of these factors, Arp2/3 and CapZ polymerize a network of branched actin proximal to the ER membrane (see **Figure 1**—nucleation; actin polymerization). This ATP-dependent and spatially controlled polymerization of actin generates pushing forces against the membrane, and thus sustaining the dome-shaped concavity therein (the isolation membrane) (45). In addition, this branched actin network provides a structural scaffold to sustain the pronounced curvature of the membranes forming the omegasome first and the subsequent phagophore. In particular, the latter would energetically tend to open into a spherical vacuole rather than keeping its typical cup shape due to the high curved edges. The preferred shape of a vesicle is defined by minimizing the membrane bending energy for a given enclosed volume (53). To overcome this energy barrier, cells take advantage of several tools such as asymmetric lipid and protein distribution between the two faces of the bilayer (e.g. PI(3)P and cholesterol) and the action scaffolding proteins (e.g. Atg17) and scaffolding cytoskeletal structures (actin) (53–56) (see **Figure 1**—nucleation). However, in a field that is disproportionally focused on protein-mediated signaling cascades, the importance of physical properties of the phospholipid bilayers has been largely overlooked. While PI(3)P and actin polymerization primes the physical environment, ULK1 also initiates a second crucial cascade leading to recruitment of phospholipids to assemble the pre-autophagosomal double membrane, which is achieved by the recruitment of vesicles receiving input from different membrane sources (mitochondria-associated ER membrane, ER, Golgi, plasma membrane, and recycling endosomes) (57, 58). This seems to be accomplished by two mechanisms: ATG9-vesicle transport and fusion with the omegasome (35) and ATG2-mediated transport of lipids from one donor compartment to the omegasome (59). Various signaling pathways such as EGF/Src induce incorporation and phosphorylation of cytosolic ATG9 in the target membrane and the formation of ATG9-vescicles (60, 61). The selectivity of the source of the membranes, depending on the type of autophagy and the nature of the cargo to be sequestered, is still debated (44). In general, intracellular membrane trafficking is regulated by the Rab family of small monomeric GTPases (62). In their GTP-bound form, Rab proteins recruit effectors to regulate vesicle trafficking, while hydrolysis of the bound GTP to GDP causes loss of effector binding and extraction from membranes. Upon activation of the autophagic process, activated Rab11/Ypt11 GTPase regulates the recruitment of ATG9 vesicles to the omegasome through the tethering of ATG9 to ULK1 (48, 49, 63). Actomyosin contractility seems to play a fundamental role in ATG9-vesicle transport. It has been shown that activation of myosin IIA *via* MLCK-like protein Sqa, which is downstream of UKL1, induces transport of ATG9 vesicles to the phagophore (64). While the proposed mechanism of cargo transport by

myosin IIA seems farfetched, as myosin IIA is not a cargo transporter (65), it is possible that cables of actin under tension provide physical guidance for the flow of vesicles toward the phagophore. Recent work presents a different mechanism for the transport of phospholipids from the donor membrane to the forming autophagosome (66). Indeed, according to the experimental evidence, Atg9 establishes membrane contact sites with a donor compartment. Here phospholipids are transferred between compartments by lipid transfer proteins like Atg2, resulting in a net flow of lipids from the vesicles to the autophagosome without vesicle fusion (66).

Elongation and Closure Stages

After priming of the physical environment, the membrane of the nascent phagophore elongates to an open cup-shaped structure thanks to the fusion of additional membrane (**Figure 1**—elongation). This novel structure encompasses a portion of the cell cytosol, which is ready to accept the material to be recycled (cargo loading) and finally seal through SNARE-mediated fusion (**Figure 1**—elongation and closure). In mechanical terms, the growth of the phagophore double membrane has the same mechanical requirements as the previous stage. Hence, this stage follows the same dynamics with lipid being recruited through ATG9-mediated fusion and/or transfer (**Figure 1**—elongation; membrane recruitment) and polymerization of actin cytoskeleton to structurally support the growing double membrane and maintain its shape (67) (**Figure 1**—elongation; actin polymerization). These processes are under strict regulation by several ATG proteins (such as ATG3, ATG7, ATG5, ATG12 and ATG16L1), including the lipidated LC3/GABARAP protein family (25). The lipidation process occurs by conjugating the cytosolic LC3-I protein to phosphatidylethanolamine (PE), which generates the membrane bound LC3-II (68–70). Importantly, LC3 lipidation requires the curved rim of the phagophore, as ATG3, the E2-like enzyme necessary for LC3 lipidation, only functions on a highly curved membrane (71). Additionally, it has been observed that the local curvature of the phagophore increases upon LC3 insertion, indicative of the curvature-inducing properties of LC3 (72). Hence, PE localization and enrichment on the phagophore inner membrane is fundamental for the progression of autophagy. Indeed, it has also been proposed that phospholipid transfer (PE precursors) from the ER to acceptor membrane on adjacent organelles (e.g. mitochondria) may be the mechanism which induces formation of the phagophore on sites other than the ER (73, 74). Among other cargo-receptors, LC3 is fundamental for selection and loading of specific cargo into the autophagosome [reviewed in (75)] (**Figure 1**—elongation; cargo loading). LC3 is also known to regulate cytoskeletal dynamics. On one hand, LC3 recruits NPFs (i.e. WHAMM and JMY) to promote the Arp2/3-mediated expansion of the membrane-proximal actin network and allow for the phagophore extension and shaping (13, 76). On the other hand, interaction of LC3 with microtubules has been proposed to mediate transport and selection of dysfunctional organelles (77), phagophore expansion and later in the process to mediate the closure of the autophagosome (16, 78, 79). Once loaded with its content, the phagophore closes into a double membrane organelle, the autophagosome proper, to confine its inner degradative space (80, 81) (**Figure 1**—closure). Prior to closure all the ATG proteins tethered to PI(3)P platform are removed from the surface of the autophagosome. This process requires the removal of PI(3)P by phosphoinositide phosphatases and possibly other factors (82–84). It must be noted that the clearance of PI(3)P is an important mechanism to dismantle the nucleating-elongating ATG machinery, required for the formation of the mature autophagosome (82). Finally, the closure of the phagophore is completed by a scission (or fission) process of the inner and outer membrane of the phagophore to generate the autophagosome with a double membrane (85). This process, still not completely understood, is mediated mainly by the endosomal sorting complex required for transport (ESCRT) (58, 86) and shares topology with canonical ESCRT-dependent cellular membrane scission processes, including cytokinesis, plasma membrane repair and multivesicular body biogenesis (87, 88). The ESCRT machinery is composed by distinct conserved complexes (ESCRT- I, -II and -II) and accessory proteins, such as ATPase protein VPS4, which disassembles and recycles ESCRT-III complex (89, 90). During the process, ESCRT-III subunits assemble into helical filaments providing the driving force to induce membrane deformation, while the recruitment of VPS4 drives membrane sealing (88) and, subsequent scission (91, 92). In addition to the ESCRT complex the motor protein Myo6 and the actin network participate in phagophore closure (87, 88). Altogether these components bring the open ends of the autophagosome in close contact to allow for SNARE-mediated fusion (93).

Maturation and Formation of Autolysosome

Once the double membrane is fused, the process of autophagosome maturation begins. This requires fusion of the autophagosome with early/late endosomes (**Figure 1**—maturation) and transport towards a perinuclear region mediated by microtubules and dynein (**Figure 1**—maturation; transport) (18, 19). This is followed by fusion of the mature autophagosome with lysosome to form the autolysosome where the degradation of cargo occurs (**Figure 1**—autolysosome formation). Autophagosome–endosome/lysosome fusion may occur by a large variety of mechanisms, including kiss-and-run, complete fusions or fusion mediated through tubules (94). In these processes, docking and fusion appear to be two separately regulated events. Once the autophagosome and the lysosome encounter, the outer membrane of the autophagosome fuses with the lysosome forming an autolysosome. The fusion of endo-lysosomal vesicles with autophagosomes broadly requires Rab GTPases for trafficking and vesicles docking (in particular Rab7), membrane-tethering complexes and SNAREs to mediate vesicles fusion in a specific manner (95–97). The molecular mechanism regulating the fusion of autophagosomes with lysosomes has not yet been fully understood. Recent evidence shows that increased levels of PI(4)P on late endosomes/lysosomes stimulate the recruitment of the multisubunit homotypic fusion and vacuole protein sorting (HOPS) complex (98). HOPS complex, by interacting with LC3, tethers lysosomes to autophagosomes (99, 100) and, by direct interaction with autophagosome-localized STX17, facilitates the

assembly of the SNARE complexes (101) between STX17 with its partners, ubiquitous SNAP29 and with lysosomal VAMP3 (95, 98). Beside the HOPS complex, TECPR1, a protein that localizes at lysosomal membranes has also been proposed as tethering factor that initiates autophagosome-lysosome fusion (102) by recruitment of LC3 matured autophagosomes to lysosomes and promoting the degradation of protein aggregates (103). The whole process of tethering and fusion is accompanied by the omnipresent actin network that stabilizes the curvatures and provides the mechanical energy to force the membranes of different organelles in close contact and fusion (11, 17). This latter process is mediated by WHAMM-dependent polymerization of branched actin network (cortactin and Arp2/3), leading to the appearance of stress-bearing actin comets (13), and by the unconventional myosin motor protein Myo1C (104). Contrary to the canonical, fast twitching myosins (e.g. Myosin IIA), Myo1C is a slow monomeric actin-based motor protein adapted for translocation of large loads at a slow pace. Though its mechanistic action is not completely elucidated, its suggested function is to link membrane cargo enriched in PI(3,5)P2 [produced by PIKfyve-dependent PI(3)P dissipation (105)] to the actin cytoskeleton (106) and to stabilize membrane ruffles (107).

Cargo Degradation and Recycling

Upon fusion of lysosomes with the autophagosome and the degradation of the inner membrane, the process of autophagosomal cargo degradation begins, as depicted in **Figure 1** - Degradation and Recycling (43). During this step the autolysosomes significantly reduces in size (108), due to cargo degradation and the transport of small solutes (amino acids, monosaccharides and nucleosides) mediated by the solute carrier transporter (109). Solute transport across the autolysosome membrane is followed by the subsequent osmotic forces causing an outward flow of water. The shrinkage of the autolysosome is required for the following step of lysosome membrane recycling. The high membrane curvature, driven by the autolysosome shrinkage, recruits the protein complexes required for the processes of vesiculation and tubulation that allow lysosomes vesicles to reform (110). Autolysosome tubulation is also facilitated by the protein WHAMM, which, once recruited in autolysosome surface, promotes the formation of a branched actin scaffold that facilitates the process (108).

CELLULAR MECHANICS AND AUTOPHAGY

A great variety of biophysical stimuli elicit cellular responses and determine cellular functions (**Figure 3A**) (111). Much of these stimuli stem from short-scale (**Figure 3A**, blue boxes) interaction between the cells and their physicochemical microenvironment. Cells have been reported to sense and respond to the a) physical status of the extracellular matrix (e.g. composition, stiffness, topography and density) by exerting traction forces on the substrate (112–114), b) geometrical cues (e.g. size, confinement, curvature) affecting cortical and membrane tension (115, 116), c) presence of surrounding cells (e.g. cell crowding) and their physical activity (e.g. pulling and pushing causing cell-cell shear and normal forces), and the chemical composition of the interstitial and luminal fluids (e.g. osmotic pressure inducing cell swelling or shrinkage and consequent variation in membrane tension) (117). In addition, cells are subject and respond to large-scale mechanical forces (**Figure 3A**, red boxes) such as shear stresses and fluid pressure due to flow of liquids or solid material in the lumen of tubular structures (e.g. gut, blood vessels and urinary tract), and lateral stretch and compression of tissues required for physiological function of lungs, muscles, and digestive system, among others (118–121). Short and long-scale force (summarized in **Figure 3A**) elicit reactive and adaptive cellular responses that primely involve active processes mediated by the cell cytoskeleton (4). This can be activated by the direct action of external mechanical cues on the cytoskeleton *via* sensing mechanisms involving various mechanosensors at the cell surface, such as mechanically activated ion channels (e.g. TRP and piezo), proteins sensing tension and curvature of the plasma membrane (e.g. BAR proteins) and of the cytoskeleton (e.g. filamin), and adhesion protein complexes (e.g. focal adhesion, adherens junctions) (122). These mechanosensors translate mechanical inputs into biochemical signals *via* mechanotransducers (AKA mechanotransduction process) that control cytoskeletal organization, membrane trafficking, gene expression profile and ultimately cellular function as a whole (4, 123, 124) (**Figure 3B**). Mechanosensing is generally achieved by a force-dependent conformational change of the sensing protein that may lead to the opening of a channel (typically calcium channels), which subsequently activates a cellular response *via* an electrochemical signal, or through the dissociation of proteins (mechanotransducer) from the sensing complex. In its freely diffusive form, the mechanotransducer participates in enzymatic reactions (e.g. phosphorylation), either as the enzyme or the substrate. As examples of both cases, the rise of calcium and/or the activation of protein phosphorylation cascades will lead to short- and long-term adaptation to mechanical stimuli. Acting as an essential part of the innate adaptive mechanisms of the cell, the autophagic response aids in the management of mechanical challenges and allows the cell to adapt to the everchanging physical environment (**Figure 3B**). In general terms, mechanical cues may affect the autophagic process in two ways: firstly, *via* specific crosstalk between mechanotransduction and autophagy regulatory proteins (e.g. mTORC, AMPK) (125, 126) responsible for the initiation and/or inhibition of the autophagic process and/or secondly, *via* the unspecific cooperation/competition mechanisms between mechanical processes and autophagy to recruit cytoskeletal elements and phospholipid membranes (127). A growing body of evidence demonstrates that indeed mechanical cues feed into the signaling required for the activation of autophagy (7, 128–130). Conversely, despite being highly plausible, the competition for cellular components between the two processes and the consequences of such, have still to be addressed by the literature. A final point of convergence is the regulatory role of autophagic

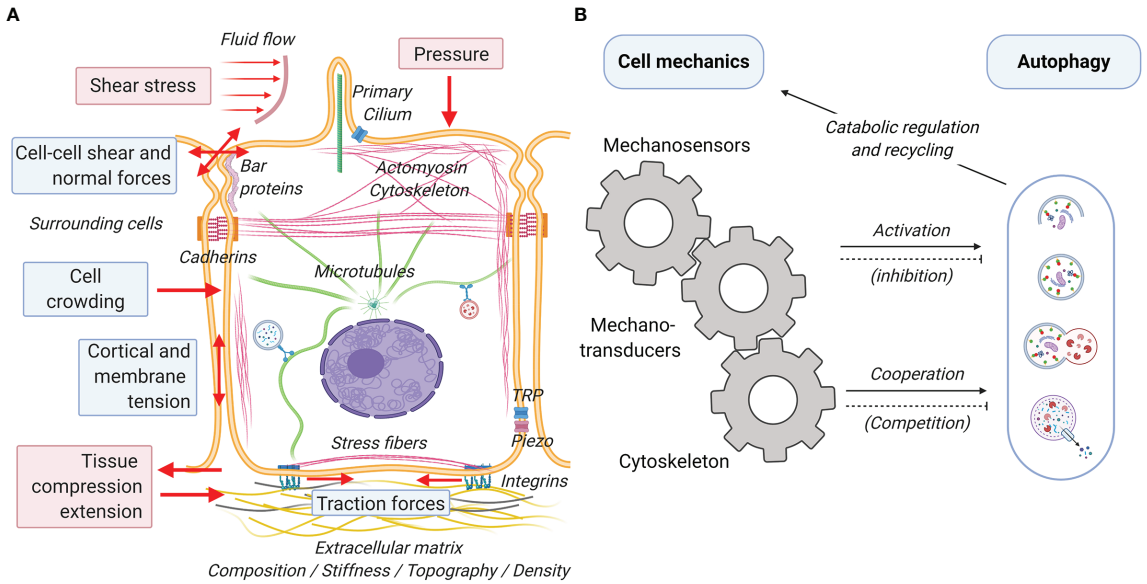


FIGURE 3 | Schematic representation of cell mechanics and its interplay with autophagy. Cells subjected to a great variety of mechanical forces (red arrows) from the environment that generate cell-autonomous forces mediated by the cytoskeleton. **(A)** There are two main different categories of forces sensed by the cells: short scale (blue boxes) and large scale (red boxes). Short and long-scale forces are perceived by the cells *via* various mechanosensors, including interfacial protein complexes (integrin- and cadherin-mediated adhesions), mechanosensitive ion channels (TRP, piezo), tension and curvature sensors at the plasma membrane and actin cortex (BAR proteins, filamin), and the primary cilium. **(B)** Mechanical inputs are transduced to biochemical signals (mechanotransducers), such as Ca^{2+}, transcription factors (YAP/TAZ) and signaling proteins (phosphatases and kinases) that affect the cytoskeleton, gene regulation, and other cellular functions. Autophagy is directly (cellular signaling mediated) and indirectly (cooperative action with the cytoskeleton) activated by mechanical processes. While likely to exist, negative feedbacks (inhibitions and competition for cytoskeletal elements) are still underexplored in the literature. Autophagy regulates various mechanical processes *via* ensuring recycling of cellular components and providing energy during catabolism.

catabolism and recycling of biological components in managing the turnover of cellular components necessary for proper execution of mechanical processes. In the following sections, we will discuss the crosstalk and interactions between cell mechanics and autophagy. Next, we will discuss in detail some the most relevant and better known connection between the mechanotransduction machinery and the autophagic process.

Extracellular Matrix and Focal Adhesions

The macromolecular composition, structural architecture, and rheological properties of the extracellular matrix undergo constant remodeling due to the enzymatic and mechanical action of the cells (113, 131). These modifications and the remodeling processes deliver a versatile microniche that in turn affects cell phenotype and function and, when dysregulated, may lead to the emergence of disease states such as fibrosis and cancer (111). The ability of cells to sense mechanical properties of the extracellular matrix in normal and in pathological conditions can be attributed to the integrin-mediated adhesions, also known as focal adhesions (132, 133). Focal adhesions are composed of multiple mechanosensors (e.g., talin, vinculin), signaling molecules (e.g., FAK, Src, PI3K), adaptor proteins (e.g. paxillin) and actin linker proteins (e.g., filamin, alpha-actinin), which physically connect the integrins to the cytoskeleton [reviewed in (134)]. The binding of extracellular matrix ligands to integrin heterodimers promotes tension-induced conformational changes in the integrin cytoplasmic tail, leading to the recruitment of talin and paxillin (135, 136). As tension increases and focal adhesion mature, protein tyrosine kinase 2 and Src are recruited, providing the enzymatic kinase activity to promote downstream signal transduction, including Rho GTPase signaling, anoikis signaling, mitogenic signaling, and extracellular matrix turnover (137). Thus, integrin-mediated adhesions interact with the extracellular matrix and sense its rigidity, which in turn modulates cellular behavior including motility and migration (138). Several studies address how the extracellular matrix and integrin-mediated adhesion may trigger autophagy *via* FAK and ILK (integrin linked kinase), thus linking it to anoikis and cancer progression (detachment-induced cell death) (139–141). Importantly, these emerging interconnections between integrin-mediated adhesion pathways and autophagy are relevant for immunosurveillance (142) and thus impinge on the appearance of certain diseases, including cancer. Matrix constituents have been shown to regulate autophagy in both a positive (activators) or negative (inhibitors) manner. Decorin, collagen VI, kringle 5, perlecan, and endostatin function as activators (142–145), whereas laminin a2 is an inhibitor of the autophagic process (146). The extracellular matrix, which constitutes different physical and structural properties, can initiate biochemical signaling cascades that involve membrane receptors (e.g. integrins, VEGFR2, GRP78) (143, 144, 147), regulatory proteins (AKT, mTORC1 and 2) and autophagy specific effectors, including VPS34, Beclin-1 and lipidated LC3 (LC3-II) (142, 148). On the

other hand, autophagy regulates integrin-mediated adhesion, and therefore cell migration, *via* controlling focal adhesion turnover through a mechanism involving LC3, paxillin and Src (149).

Cell–Cell Adhesions

In addition to the extracellular matrix, cells in a tissue physically interact with other cells (e.g. epithelial cells, muscle cells) through transmembrane receptors that mediate extracellular bonds with receptors on neighboring cells to control tissue integrity and collective cell dynamics (150). Cell-cell contacts are mediated by various adhesion complexes, such as adherens junctions, tight junctions and desmosomes, each with distinct functions and molecular characteristics. Adherens junctions are force-sensor complexes. Tight junctions only appear to act in parallel to adherens junctions *via* a physical connection between the two complexes. The role of the desmosome in junctional mechanotransduction responses remains elusive. In adherens junctions, coupling between the cadherin transmembrane receptor and the actin cytoskeleton is mediated by a protein complex collectively termed the cadhesome network (151). Similarly to the previously described integrin-based adhesion, this complex has a well-defined spatial organization where force-transduction is mediated by protein conformation that in turn modulates the engagement of cadherins with the actin network (152, 153). Tension at adherens junctions induces an α-catenin conformational switch with consequent exposure of previously hidden binding sites for vinculin, resulting in increased functional integration of the complex with actin dynamics. Tension-induced conformation changes of vinculin can differentially engage the signaling layer to the actomyosin contractile machinery and enable localized actin polymerization through the Mena–VASP complex associated with vinculin (152, 153). Thus, vinculin serves the role of 'molecular clutch' that integrates mechanical and biochemical signals to engage and disengage the cell-cell junction to internal and external forces. This remarkable spatial organization and the molecular mechanism involved therein provide the cells with the strength and plasticity needed by the highly dynamic epithelial tissues during biological processes such as collective cell migration, wound healing, tissue stretching, etc. Autophagy plays a critical role in junctional homeostasis by actively regulating the recycling of the junctional complexes in response to various intra- and extra-cellular cues [reviewed in (154)]. Experimental evidence shows an autophagy-dependent translocation of cadherin (155) and claudin (156) from the cell membrane to the cytosol where they are subsequently degraded by the autophagosome or lysosome. The effect of cell-cell adhesion on the autophagic process has been less well studied. Nevertheless, it has been shown that the application of force to E-cadherin stimulates autophagy *via* Liver Kinase B1 (LKB1) activation, which recruits the autophagy-initiator-factor AMPK to the E-cadherin complex (157).

Yes-Associated Protein/Transcriptional Co-Activator With PDZ-Binding Motif Signaling

In addition to what has been discussed in previous paragraphs, autophagy, and mechanosensing are interdependent *via* the YAP/TAZ system. Yes-associated protein (YAP) and the transcriptional co-activator with PDZ-binding Motif (TAZ) regulate gene expression in a force-dependent manner. Pioneering work of Piccolo and co-workers showed that mechanical forces regulate YAP/TAZ cytosolic localization and nuclear translocation (158). By analyzing YAP localization and transcriptional response, these investigators showed YAP activity to be regulated by extracellular matrix stiffness, cell density and cell geometry. When cells are at low density or on a stiff extracellular matrix, YAP and TAZ are active and localize in the nucleus, where they interact with the DNA-binding transcription factor TEAD to promote the expression of several growth-related genes and ultimately induce cell proliferation (159, 160). Conversely, when cells are at high-cell density or plated on soft matrix, YAP/TAZ are inactive in the cytoplasm (158, 161, 162) leading to contact inhibition of proliferation. This force-dependent control of proliferation is a fundamental mechanism to maintain tissue homeostasis and allow tissue repair. Impairment of this system may lead to uncontrolled cell growth (a cancer hallmark). Interestingly, one of the transcriptional targets of YAP/TAZ is Armus (163), a protein of the Rab-GAP family that mediates autophagosome-lysosome fusion (164). Consequently, it has been seen that the efficiency of the autophagic flux depends on the physical properties of the cell microenvironment *via* YAP/TAZ mechanical response (163). Furthermore, mTORC1 regulates YAP by mediating its autophagic degradation (165), further linking cellular nutrient status to YAP activity (166).

Mechanosensitive Ion Channels

Calcium influx mediated by mechanosensitive channels have been implicated in the initiation and elongation stages of autophagy (167). ER-resident channels exhibit the potential to regulate autophagy at different stages (initiation as well as the autophagosome-lysosome fusion), due to its special role as a platform for autophagosome nucleation. However, these ER-resident channels have not being linked to mechanosensing. On the other hand, plasma membrane channels have been reported to control the initiation process *via* AMPK and mTOR. Interestingly two large families of calcium channels, the (osmo-mechano and voltage) transient receptor potential (TRP) channels and pore-forming Piezo (168) are known to be mechanosensitive. These two channel families are gated by changes in membrane tension, which may arise from stretch and compression of the plasma membrane during cell migration or when the cells are subject to shear flow. Similarly, these channels respond to osmotic challenges which increase membrane tension during cellular swelling (169).

Membrane and Cytoskeletal Tensions

As a physical boundary between the cell and the environment, the plasma membrane constitutes a prime location for mechanosensation and mechanotransduction (170, 171). The poorly extensible lipid bilayer (rupture occur at only 3–5% area expansion) is mechanically supported by the actin cortex, which, thanks to its active dynamics, absorbs a great portion of applied stress, control folding and unfolding of plasma membrane into and out of membrane reservoirs and facilitates vesicle trafficking and fusion. Mechanical stimuli at the plasma membrane can be differentiated as tensile stress (cell stretching and hypoosmotic swelling), compressive stress (cell compression and hyperosmotic shrinkage), shear stress (flows of fluids over adherent cells) and

forces generated by topographical cues (confinement caused by the physical microenvironment). Fluid shear stress has been reported to induce autophagy by activating the Rho GTPases (Rac1, RhoA, and Cdc42) with consequent upregulation of Beclin-1, ATG5, ATG7 and LC3 (172). Furthermore, cells respond to mechanical stress with rapid autophagosome formation through an mTOR-independent pathway (173). Autophagic response demonstrates high specificity to mechanical load with a transient and gradual response to the stimulus (half-maximal responses at ~0.2 kPa) (173). While the exact sensing and signaling mechanisms are not entirely clear, they may involve BAR proteins that have been identified as primary membrane tension sensors (174). Another mechanism of tension sensing involves the actin scaffold protein filamin. Filamin A control the tensional state of the actin cytoskeleton by mediating crosslinking of actin filaments at large angles (175, 176). When cells are challenged by sheer flow, filamin accumulates throughout the cell, increasing the overall mechanical stability of the cytoskeleton (177). In addition, filamin A crosslinks integrin with actin and thus mediates force-dependent reinforcement at the focal adhesions (178). In response to tension, filamin A undergoes conformational changes that promote its ubiquitination and subsequent targeting by chaperone-mediated autophagy (179).

Mechanosensing at the Primary Cilium

Key processes such as cell migration, differentiation, cell cycle re-entry and apoptosis largely depend on the specific activity of the primary cilium (180). Found in the majority of cell types, the primary cilium is a non-motile microtubule-based appendix that senses extracellular chemical and mechanical stimuli (181, 182). For instance, the cilium in kidney cells is a flow sensor. Sheer forces causing bending of the the cilium induce calcium entry into the cell *via* polycystin-2 (PC2) and transient receptor potential vanilloid 4 (TRPV4) (183). This sheer-stress-dependent signaling triggers autophagy and leads to cell size regulation (184, 185) through the LKB1-AMPK-mTOR signaling pathway (186). In contrast to starvation-induced autophagy, mechanical signaling from the cilium initiates autophagy in a ULK1, Beclin-1 and PI3K/VPS34 independent manner (187, 188). It was recently reported that the PI3KC2α lipid kinase (PI3K class II), required for ciliogenesis and cilium function, can promote the synthesis of a local pool of PI(3)P in response to shear stress (188). In turn PI(3)P is crucial for Rab11a membrane mobilization and activation (189, 190), and serves as platform for autophagosome assembly and formation (191, 192). On the other hand, primary cilium length and assembly (ciliogenesis) are modulated by autophagy. This involves the degradation of ciliogenesis regulators (193, 194), as evidenced that during starvation, several components of the autophagic machinery (including ATG16L1) localize at the cilium's basal body (40).

MECHANICS OF AUTOPHAGY DURING CANCER TRANSFORMATION

Malignant transformation is accompanied by a progressive loss of tissue homeostasis and perturbations of tissue architecture. It is widely recognized that a critical component of this transformation involves alterations in the mechanical phenotype of the cell and of the surrounding microenvironment, creating a peculiar mechanical milieu predominantly composed of cancer cells surrounded by a dense extracellular matrix (6, 195, 196). In addition, a set of accessory cells may be found in the tumor microenvironment, including blood and lymphatic vascular cells, lymphocytes, inflammatory cells and cancer associated fibroblasts (6, 195, 197). Depending on the context and stage of cancer development, autophagy has been recognized as a "double-edged sword" (**Figure 4**, left panel), as it can act as a mechanism for either tumor-suppression or tumor-promotion depending on the cellular context in which it acts (198, 199). Consistent with its role in promoting cell survival and rejuvenating cellular components, autophagy serves as a quality-control mechanism, detecting changes in organelle architecture and protein folding and thus preventing tumor initiation. On the other hand, these same mechanisms promote cancer cell survival and escape from apoptosis. This occurs by aiding the responses against environmental stress and generating the energy needed for unregulated growth and metastasis through the recycling and degradation of cellular organelles (**Figure 4**) (198, 200–202).

In the context of solid tumors, several mechanical aspects of the tumor microenvironment contribute to the tumor-promoting function of autophagy (**Figure 4**, right panel). When confined by the extracellular matrix, cancer spheroids experience forces exerted by the expanding tumor mass as a result of unchecked proliferation and the resistance to deformation of the surrounding stromal tissue (203, 204). This causes increased interstitial pressure (203) and generates shear stress within the tumor microenvironment (7, 205, 206). Eventually, this mechanical stress affect cell growth directly, by compressing cancer cells, and/or indirectly, by compressing the surrounding blood and lymphatic vessels (207). Due to the sustained compression of the vasculature within the tumor, poor tissue perfusion causes hypoxia and eventually necrosis within the tumor (208). Hypoxia promotes epithelial to mesenchymal transition (EMT), a reorganization of the cytoskeleton and dissolution of the epithelial cell-cell junctions. This enables dynamic cell elongation, directional motility (209), and consequently an increase in the metastatic potential of the tumor cells. Furthermore, during EMT the composition of intermediate filaments changes, switching from keratin to vimentin (195, 210). Furthermore, during this process the actin cytoskeleton becomes hypercontractile through the TGF-β-dependent activation of pathways such as Rho GTPases, p38MAPK and ERK1/2 (211). This pathway activation triggers actin reorganization and formation of cellular protrusions, including lamellipodia and filopodia (212, 213). Furthermore, TGF-β and hypoxia also promote the formation of cancer associated fibroblasts (214, 215), which interact with each cellular component of the tumor microenvironment.

By mediating extracellular matrix stiffness, the cancer associated fibroblasts can regulate the cancer cell cytoskeleton (216–218). These changes to the cellular cytoskeleton during transformation or EMT drastically alters their mechanical

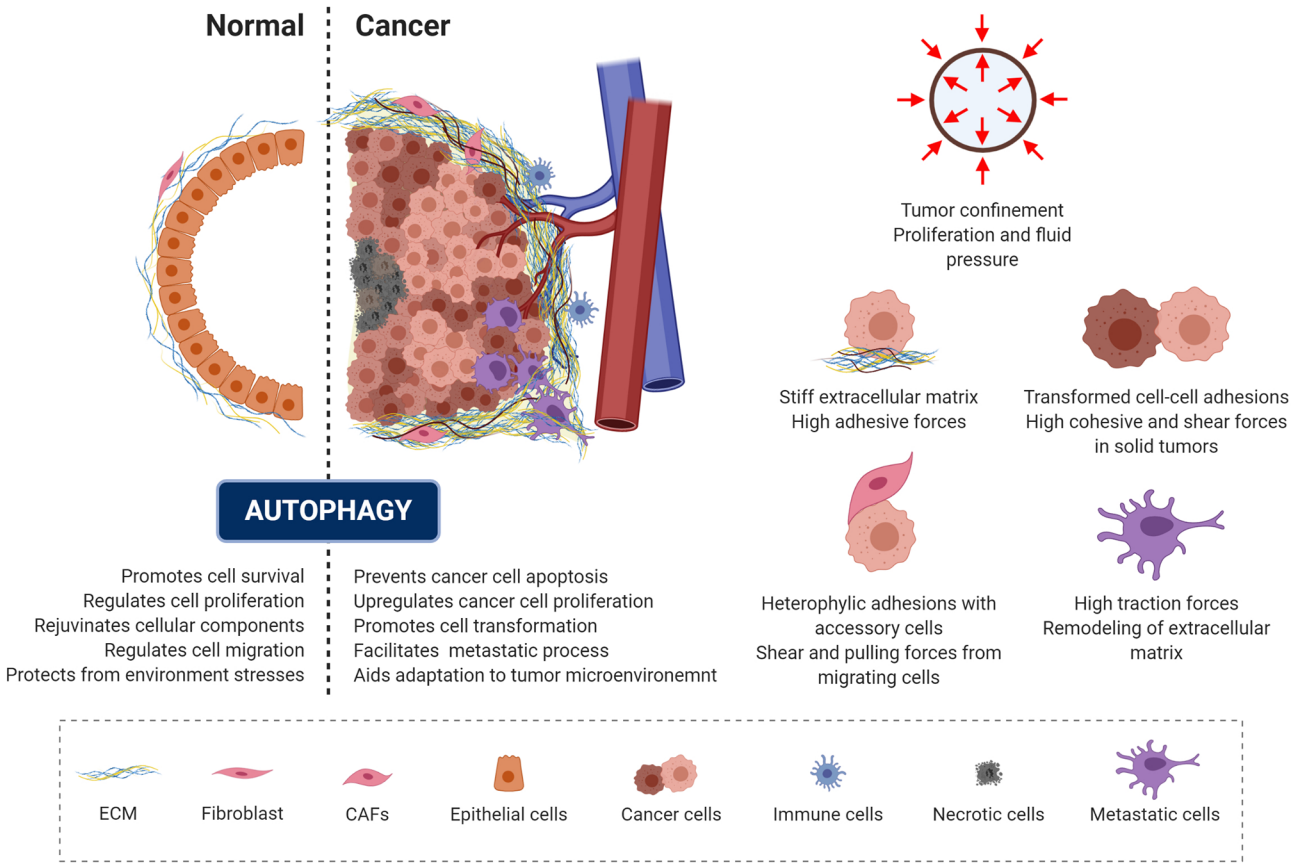


FIGURE 4 | Autophagy and mechanics during cancer transformation. The role of autophagy in preserving cell homeostasis and protecting cells from mechanical environmental stress is represented and described in the left panel (dotted line separates the normal and cancer context). Cancer cells exploit autophagy to adapt to the tumor microenvironment and promote malignant progression. Right panel illustrate the mechanical components of the tumor microenvironment.

phenotype and in particular the degree of tension exerted on neighboring cells and the extracellular matrix, leading to increased migration, invasion and dissemination potential (201, 219). To successfully metastasize, tumor cells migrate locally and invade into surrounding tissue to gain vasculature access, and subsequently intravasate through the basal membrane and detach from the extracellular matrix to become circulating tumor cells (220, 221). Ultimately, circulating tumor cells that survive in circulation can extravasate from the bloodstream and engraft in secondary tissue sites and thus forming metastatic foci (222). All cells that travel in the bloodstream experience fluctuating levels of shear stress. Hemodynamic shear stress, caused by the movement of blood along the cell surface, is influenced by both the fluid viscosity and fluid flow velocity (210). Shear stress can also be caused by frictional interaction with endothelial cells (6, 223). Equally, tumor cells within the bloodstream must survive harsh conditions, including extracellular matrix detachment-induced apoptosis (i.e. anoikis), immune system assault, along with the variations in shear stress (222). The physiological shear stress (0.5–3 Pa) caused by blood flow may suppress cancer cell proliferation but may also promote migration and adhesion (224–227). Substantial evidence suggests that mechanical stress, such as compressive and shear forces, in the tumor milieu boosts malignant progression by inducing autophagy (129, 130, 172, 228). Consistent with this, cervical cancer cells exposed to pulses of laminar shear stress of 2 Pa (over 3 and 6 minutes) undergo autophagy, by a lipid raft-mediated p38MAPK dependent process, and delay apoptotic cell death (130). However, shear stress is not necessarily beneficial to the cancer cell. Conversely, elevated levels of shear stress (6 Pa), as occurs during intense exercise, has been shown to promote tumor cell death (229). Furthermore, fluid shear stress in the range of 0.05 to 1.2 Pa is shown to trigger cancer cell death through apoptosis and autophagy in several cancer cell lines, including hepatocarcinoma, osteosarcoma, oral squamous carcinoma, and carcinomic alveolar basal epithelia. Interestingly, this fluid shear stress induced death did not occur in non-cancerous cells (230). Thus, it appears that depending on both the intensity and the duration of the shear stress, autophagy may act as either a pro- or anti-survival mechanism. Furthermore, it has been shown that autophagy induced by shear or compressive stress plays a role in cytoskeletal remodeling and in the recycling of proteins essential for cancer progression (149, 172). Indeed,

increased tissue stiffness is implicated in the control of several tumor features, such as growth, invasion, and metastasis (203, 231, 232). Accordingly, it has been observed that the stiffness of cancerous tissue of breast, hepatic and liver origin is higher than that of the corresponding respective physiological context (233–236). Extracellular matrix stiffening in tumors is produced as consequence of stroma reorganization, through the excessive activity of extracellular matrix proteins and enzymes that covalently cross-link collagen fibers and other extracellular matrix components (237, 238). Collagen crosslinking enhances integrin activation, focal adhesion maturation, intracellular contraction and thus causes a subsequent increase in the stiffness of the actin cytoskeleton, which may favor cancer cell migration and invasion (214, 239–241). The higher extracellular matrix stiffness also plays a role in the onset of the malignant phenotype: cytoskeletal tension leads to increased cell-extracellular matrix adhesions and disruption of cell-cell junctions (242). Enhanced collagen deposition in the extracellular matrix leads to activation of the Hippo signaling pathway (159, 162, 243) with a consequent loss of contact inhibition. Autophagy is also reported to have a pivotal role at the center of these processes. Autophagy is reported to be compromised in contact-inhibited cells in both 2D or 3D-soft extracellular matrix cultures. In such cells, YAP/TAZ (previously mentioned to be regulated by mechanical forces) fail to co-transcriptionally regulate the expression of myosin-II genes, resulting in the loss of F-actin stress fibers, which leads to impairment in autophagosome formation. This loss of F-actin stress fibers is also associated with a reduction in the number of ATG16L1 puncta per cell and with decreased co-localization of ATG9A-LC3, suggesting an alteration in the trafficking of key autophagy proteins and thus a defective autophagic response (244). Furthermore, compressive stress-induced autophagy can promote secretion of matrix metalloproteinase-2 and the turnover of the focal adhesion paxillin, boosting the invasiveness of the HeLa cervical cancer cell line (129). In line with these results, it has been suggested that paxillin binds directly to LC3 to stimulate focal adhesion disassembly in MDA-MB-231 human breast cancer and in B16.F10 mouse melanoma cell lines, and furthermore promote metastasis *in vivo* in the 4T1 mouse mammary tumor model (149). Another mechanism of force sensing in cancer involves filamin A. This actin and actin-integrins crosslinker is down-regulated in human bladder cancer, reducing autophagy in these cancer cells, as indicated by the decrease in the levels of LC3-II and decrease in LC3-I (245). It has been further reported that upon overexpression, filamin A attenuates autophagy and suppresses the invasive ability in cancer cells. The mechanism of action may involve the inhibition of matrix metalloproteinases expression, regulation of integrin function and enhances apoptosis (245–247). Interestingly, YAP/TAZ signaling has been shown to stimulate filamin A transcription to maintain actin anchoring and crosslinking under mechanical tension (248). This could be a potential mechanism for cancer cells, to control autophagy through a crosstalk between YAP/TAZ and cytoskeletal elements. Low mechanical stress has been shown to activate Caveolin-1, triggering the FAK/Src and ROCK/p-MLC pathways, which are involved in the reorganization of the cytoskeleton, cell motility, focal adhesion dynamics and breast cancer cell adhesion (227). PI3K/AKT activation and β-Catenin-TCF/LEF-dependent activity downstream from Caveolin-1 also correlates to increased VEGF expression and thus greater angiogenic potential of tumor (249). Shear stress-induced Caveolin-1 activation can induce PI3K/AKT/mTOR signaling and metalloprotease activity, which have been shown to promote cell motility and metastasis of breast carcinoma cells (250). Conversely, it was determined that phosphorylated Caveolin-1 functions to activate autophagy through binding to the Beclin-1/VPS34 complex under oxidative stress and to protect against ischemic damage (251). These data suggest that Caveolin-1 function might be cell-context dependent (252), resulting in different autophagic outcomes. Interestingly, similarly to autophagy, Caveolin-1 has also been implicated both in tumor suppression and progression (253, 254). Although potentially protective in bourgeoning tumors, higher levels of either Caveolin-1 mRNA or protein have been reported in varying cancers strongly correlating with poor survival in advanced cancer patients (227). This further implies that Caveolin-1 has a role in the metastatic process, as evidenced by increased migration, invasion and anchorage-independent growth (255). Furthermore, recent studies have unveiled the existence of an interplay between the primary cilium and autophagy in the regulation of cancer development and progression (256–258). In addition to being considered as a survival mechanism in tumorigenesis, excessive accumulation of autophagosomes may induce autophagic cell death or apoptosis (259–262), which, in the context of cancer, limits tumor growth and spread. Recently, Wang and collaborators showed that acute shear stress (10 Pa for 60 min) promotes autophagosome accumulation, which is accompanied by increased fusion of autophagic vesicles with multivesicular bodies, and reduction of autophagosome-lysosome fusion, in HeLa and MDA-MB-231 cell lines (263). Furthermore, the inhibition of autophagosome degradation, induced by mechanical stress, is associated with increased release of autophagic components in extracellular nanovesicles, possibly through a Ca^{2+}-dependent pathway involving autophagy, multivesicular bodies and exosomes (263). Thus, exosome secretion might provide a supplementary pathway to maintain cellular homeostasis when the autophagy pathway is damaged or insufficient to degrade large amounts of damaged proteins and prevent cell death (263). In conditions of mechanical stress, these results suggest a possible crosstalk between degradative and secretory autophagy to maintain cellular homeostasis and tumor cell survival (264). Furthermore, following pathological stress, harmful nucleic acids, molecular chaperones, cytosolic proteins, and misfolded proteins are released into the extracellular space through exosomes and may contribute to tumor progression and metastasis (265, 266).

As we have highlighted in the previous sections, the relation between cell mechanics and autophagy goes two ways. In the context of cancer, autophagy regulates multiple metastasis-related signaling pathways associated with cell mechanics depending on

cell type and tumor microenvironment. Autophagic protein LC3-II mediates the targeted degradation of focal adhesion proteins such as Src and paxillin (149, 267) to promote focal adhesion disassembly and turnover and lead to cell migration. Furthermore, integrins can be differently recycled and degraded, depending on their conformation, activation by ECM proteins, and binding of effector proteins, such as TLNs and FERMTs/kindlins. Integrins trafficking, recycling and degradation affect their availability at the plasma membrane, focal adhesion dynamics and Rho GTPase-mediated cytoskeleton remodeling to facilitate cell motility (127, 268). While nutrient starvation (269) increases integrin internalization and ECM degradation (270), hypoxia (271) promotes recycling of specific integrin. Since nutrient starvation and hypoxia are both hallmarks of tumor microenvironment, further investigation into how autophagy regulates integrin trafficking may provide insight into the overall role of autophagy in cancer metastasis and lead to the understanding of how microenvironmental stress act on cell mechanics to induce cancer cell exit from the primary tumor.

MECHANOBIOLOGY OF AUTOPHAGY IN CANCER TREATMENT AND IN AVOIDANCE OF CHEMORESISTANCE

In the last decade a plethora of new treatments has been introduced that significantly improved the survival of cancer patients. Despite this, highly aggressive cancers often develop primary or acquired resistance that finally cannot be treated. To this aim, new therapeutic approaches are required to overcome drug resistance and improve treatment response. Based on the reviewed literature, considering the "mechanobiology of autophagy" might represent a novel and promising approach. Indeed, even if, to the best of our knowledge, to date there are no drugs approved by the FDA or currently being investigated in clinical trials that consider the mechanobiology of autophagy in their approach there are examples of proteins/pathways that are modulated by mechanical forces thus affecting autophagy.

As previously mentioned, a pathway that is activated in cancer cells, which is regulated by mechanical forces, is the Hippo–YAP/TAZ pathway, whose inhibition has been shown promising results in reducing therapy resistance [for recent reviews see (272, 273)]. Interestingly, blockade of this pathway also reduces autophagy (244), which is targeted by several drugs currently under investigation in clinical trials, suggesting that dual inhibition of YAP-TAZ pathway and autophagy could improve treatment response. Mechanics also regulate epidermal growth factor receptor (EGFR), a protein that is regularly amplified or mutated in glioblastomas, and where autophagy is enhanced promoting cell survival (274). Inhibition of autophagy, in addition to radiotherapy, already showed positive results which might be further improved by considering the mechanics of cancer. Consistently, inhibition of Janus-associated kinase (JAK) by Ruxolitinib, a drug currently used in myeloproliferative neoplasms which inhibits cell contractility, preventing signaling downstream of focal adhesions, has recently been shown to induce autophagy (275), thus a combination of ruxolitinib with pharmacological inhibitors of autophagy needs to be followed for cancer treatment. Another drug that is currently being studied in clinical trials is losartan, an angiotensin II receptor blocker, which reduces intratumoral interstitial fluid pressure in solid tumors (276). Interestingly, this drug has also been shown to inhibit autophagy promoting autophagic cell death in cancer cells (277), again confirming the importance of targeting autophagy and mechanobiology in cancers.

CONCLUDING REMARKS

In recent years, autophagy has emerged as one of the key regulators of cellular, tissue, and organism homeostasis. Vibrant research in this field has brought to light the intricacies of autophagy's molecular machinery, together with its biochemical regulation and biomedical consequences associated with its impairment. The complex mechanobiology regulating cellular mechanical and biochemical processes is also a bourgeoning field. In this review, we hoped to bring to light the role of physical forces in autophagy regulation and their potential implications in both physiological as well as pathological conditions. More importantly, we hoped to raise questions to help investigate the mechanical requirements of autophagy and appreciate the extent to which mechanical signals affect this process. For instance, a diet rich in saturated fatty acids can negatively impact autophagic flux in neurons (278, 279). Interestingly, as the steric conformation of these phospholipids is known to mechanically decrease membrane bending, it could consequently impair autophagy by preventing vesicle fusion. However, the mechanical role of phospholipids is largely overlooked in the literature and it could represent an important area for future investigation. Similarly, to provide new frontiers for exploration, areas worthy of investigation are the action of cytoskeletal dynamics, the mechanical interplay between cellular processes, and the role of environmental cues. To achieve this a paradigm shift is required, one that adopts modern interdisciplinary approaches combining cell biology, physics, and engineering (280). To this end, cutting-edge techniques such as superresolution microscopy and the control of the mechanochemical environment (281) (e.g. by incorporating biomimetic substrates and microfluidics) will open exciting opportunities and perspectives. Combined, these technological and conceptual new directions will lead to a better understanding of autophagy and mechanisms onsetting related diseases, which in turn would pave the way to the identification of new pharmacological targets.

AUTHOR CONTRIBUTIONS

MH-C, LM, PL, AR, and CB prepared the figures. JP and FP investigated the intersection of autophagy and cell mechanics in the literature by data mining (Cytoscape). MH-C, LM, JP, FP, PL, PA, GO, EM, AR, and CB reviewed the literature. MH-C, LM, GO, EM, AC, AR, and CB wrote the manuscript. GO, EM, AR, and CB proofed the manuscript. AR and CB conceptualized and supervised the work. All authors contributed to the article and approved the submitted version.

FUNDING

AR and CB are supported by ANID PIA192015; EM and AC by ANID PIA 172066; GO by CONICYT FONDAP-15130011, IMII P09/016-F, and FONDECYT 1180241; EM by FONDECYT 1200499; AC by FONDECYT 1171075 and FONDAP no 15130011. AR acknowledges PUENTE 004/2019 and 012/2020 from the Pontificia Universidad Católica de Chile. LM, PL, JP, and FP acknowledge IPREint20 from the Pontificia Universidad Católica de Chile.

ACKNOWLEDGMENTS

All figures were created with BioRender.com.

REFERENCES

1. Collins FS, Green ED, Guttmacher AE, Guyer MSInstitute USNHGR. A vision for the future of genomics research. *Nature* (2003) 422(6934):835–47. doi: 10.1038/nature01626
2. Eskelinen EL, Saftig P. Autophagy: a lysosomal degradation pathway with a central role in health and disease. *Biochim Biophys Acta* (2009) 1793(4):664–73. doi: 10.1016/j.bbamcr.2008.07.014
3. Holle AW, Engler AJ. More than a feeling: discovering, understanding, and influencing mechanosensing pathways. *Curr Opin Biotechnol* (2011) 22 (5):648–54. doi: 10.1016/j.copbio.2011.04.007
4. Iskratsch T, Wolfenson H, Sheetz MP. Appreciating force and shape-the rise of mechanotransduction in cell biology. *Nat Rev Mol Cell Biol* (2014) 15 (12):825–33. doi: 10.1038/nrm3903
5. Chaudhuri PK, Low BC, Lim CT. Mechanobiology of Tumor Growth. *Chem Rev* (2018) 118(14):6499–515. doi: 10.1021/acs.chemrev.8b00042
6. Northcott JM, Dean IS, Mouw JK, Weaver VM. Feeling Stress: The Mechanics of Cancer Progression and Aggression. *Front Cell Dev Biol* (2018) 6:17. doi: 10.3389/fcell.2018.00017
7. Das J, Chakraborty S, Maiti TK. Mechanical stress-induced autophagic response: A cancer-enabling characteristic? *Semin Cancer Biol* (2019) 66:101–9. doi: 10.1016/j.semcancer.2019.05.017
8. Mowers EE, Sharifi MN, Macleod KF. Autophagy in cancer metastasis. *Oncogene* (2017) 36(12):1619–30. doi: 10.1038/onc.2016.333
9. Banerjee S, Gardel ML, Schwarz US. The Actin Cytoskeleton as an Active Adaptive Material. *Annu Rev Condens Matter Phys* (2020) 11(1):421–39. doi: 10.1146/annurev-conmatphys-031218-013231
10. Aguilera MO, Beron W, Colombo MI. The actin cytoskeleton participates in the early events of autophagosome formation upon starvation induced autophagy. *Autophagy* (2012) 8(11):1590–603. doi: 10.4161/auto.21459
11. Kast DJ, Dominguez R. The Cytoskeleton-Autophagy Connection. *Curr Biol* (2017) 27(8):R318–R26. doi: 10.1016/j.cub.2017.02.061
12. Chhabra ES, Higgs HN. The many faces of actin: matching assembly factors with cellular structures. *Nat Cell Biol* (2007) 9(10):1110–21. doi: 10.1038/ncb1007-1110
13. Kast DJ, Zajac AL, Holzbaur EL, Ostap EM, Dominguez R. WHAMM Directs the Arp2/3 Complex to the ER for Autophagosome Biogenesis through an Actin Comet Tail Mechanism. *Curr Biol* (2015) 25(13):1791–7. doi: 10.1016/j.cub.2015.05.042
14. Zientara-Rytter K, Subramani S. Role of actin in shaping autophagosomes. *Autophagy* (2016) 12(12):2512–5. doi: 10.1080/15548627.2016.1236877
15. Tumbarello DA, Waxse BJ, Arden SD, Bright NA, Kendrick-Jones J, Buss F. Autophagy receptors link myosin VI to autophagosomes to mediate Tom1-dependent autophagosome maturation and fusion with the lysosome. *Nat Cell Biol* (2012) 14(10):1024–35. doi: 10.1038/ncb2589
16. Geeraert C, Ratier A, Pfisterer SG, Perdiz D, Cantaloube I, Rouault A, et al. Starvation-induced hyperacetylation of tubulin is required for the stimulation of autophagy by nutrient deprivation. *J Biol Chem* (2010) 285 (31):24184–94. doi: 10.1074/jbc.M109.091553
17. Nakamura S, Yoshimori T. New insights into autophagosome-lysosome fusion. *J Cell Sci* (2017) 130(7):1209–16. doi: 10.1242/jcs.196352
18. Jordens I, Fernandez-Borja M, Marsman M, Dusseljee S, Janssen L, Calafat J, et al. The Rab7 effector protein RILP controls lysosomal transport by inducing the recruitment of dynein-dynactin motors. *Curr Biol* (2001) 11 (21):1680–5. doi: 10.1016/S0960-9822(01)00531-0
19. Kimura S, Noda T, Yoshimori T. Dynein-dependent movement of autophagosomes mediates efficient encounters with lysosomes. *Cell Struct Funct* (2008) 33(1):109–22. doi: 10.1247/csf.08005
20. Yang Y, Feng LQ, Zheng XX. Microtubule and kinesin/dynein-dependent, bi-directional transport of autolysosomes in neurites of PC12 cells. *Int J Biochem Cell Biol* (2011) 43(8):1147–56. doi: 10.1016/j.biocel.2011.04.007
21. Bahrami AH, Lin MG, Ren X, Hurley JH, Hummer G. Scaffolding the cup-shaped double membrane in autophagy. *PloS Comput Biol* (2017) 13(10): e1005817. doi: 10.1371/journal.pcbi.1005817
22. Goldman RD, Cleland MM, Murthy SN, Mahammad S, Kuczmarski ER. Inroads into the structure and function of intermediate filament networks. *J Struct Biol* (2012) 177(1):14–23. doi: 10.1016/j.jsb.2011.11.017
23. Biskou O, Casanova V, Hooper KM, Kemp S, Wright GP, Satsangi J, et al. The type III intermediate filament vimentin regulates organelle distribution and modulates autophagy. *PloS One* (2019) 14(1):e0209665. doi: 10.1371/journal.pone.0209665
24. Lowery J, Kuczmarski ER, Herrmann H, Goldman RD. Intermediate Filaments Play a Pivotal Role in Regulating Cell Architecture and Function. *J Biol Chem* (2015) 290(28):17145–53. doi: 10.1074/jbc.R115.640359
25. Al-Bari MAA. A current view of molecular dissection in autophagy machinery. *J Physiol Biochem* (2020) 76(3):357–72. doi: 10.1007/s13105-020-00746-0
26. Hurley JH, Young LN. Mechanisms of Autophagy Initiation. *Annu Rev Biochem* (2017) 86:225–44. doi: 10.1146/annurev-biochem-061516-044820
27. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol* (2011) 13(2):132–41. doi: 10.1038/ncb2152
28. Case N, Thomas J, Sen B, Styner M, Xie Z, Galior K, et al. Mechanical regulation of glycogen synthase kinase 3beta (GSK3beta) in mesenchymal stem cells is dependent on Akt protein serine 473 phosphorylation via mTORC2 protein. *J Biol Chem* (2011) 286(45):39450–6. doi: 10.1074/jbc.M111.265330
29. Sen B, Xie Z, Case N, Thompson WR, Uzer G, Styner M, et al. mTORC2 regulates mechanically induced cytoskeletal reorganization and lineage selection in marrow-derived mesenchymal stem cells. *J Bone Miner Res* (2014) 29(1):78–89. doi: 10.1002/jbmr.2031
30. Tsuji-Tamura K, Ogawa M. Inhibition of the PI3K-Akt and mTORC1 signaling pathways promotes the elongation of vascular endothelial cells. *J Cell Sci* (2016) 129(6):1165–78. doi: 10.1242/jcs.178434
31. Jhanwar-Uniyal M, Amin AG, Cooper JB, Das K, Schmidt MH, Murali R. Discrete signaling mechanisms of mTORC1 and mTORC2: Connected yet apart in cellular and molecular aspects. *Adv Biol Regul* (2017) 64:39–48. doi: 10.1016/j.jbior.2016.12.001
32. Conciatori F, Ciuffreda L, Bazzichetto C, Falcone I, Pilotto S, Bria E, et al. mTOR Cross-Talk in Cancer and Potential for Combination Therapy. *Cancers (Basel)* (2018) 10(1):23. doi: 10.3390/cancers10010023
33. Thompson WR, Guilluy C, Xie Z, Sen B, Brobst KE, Yen SS, et al. Mechanically activated Fyn utilizes mTORC2 to regulate RhoA and adipogenesis in mesenchymal stem cells. *Stem Cells* (2013) 31(11):2528–37. doi: 10.1002/stem.1476
34. Kuo JC. Focal adhesions function as a mechanosensor. *Prog Mol Biol Transl Sci* (2014) 126:55–73. doi: 10.1016/B978-0-12-394624-9.00003-8
35. Karanasios E, Stapleton E, Manifava M, Kaizuka T, Mizushima N, Walker SA, et al. Dynamic association of the ULK1 complex with omegasomes

during autophagy induction. *J Cell Sci* (2013) 126(Pt 22):5224–38. doi: 10.1242/jcs.132415
36. Graef M, Friedman JR, Graham C, Babu M, Nunnari J. ER exit sites are physical and functional core autophagosome biogenesis components. *Mol Biol Cell* (2013) 24(18):2918–31. doi: 10.1091/mbc.e13-07-0381
37. Hollenstein DM, Kraft C. Autophagosomes are formed at a distinct cellular structure. *Curr Opin Cell Biol* (2020) 65:50–7. doi: 10.1016/j.ceb.2020.02.012
38. Yim WW, Mizushima N. Lysosome biology in autophagy. *Cell Discovery* (2020) 6:6. doi: 10.1038/s41421-020-0141-7
39. Hamasaki M, Furuta N, Matsuda A, Nezu A, Yamamoto A, Fujita N, et al. Autophagosomes form at ER-mitochondria contact sites. *Nature* (2013) 495 (7441):389–93. doi: 10.1038/nature11910
40. Pampliega O, Orhon I, Patel B, Sridhar S, Diaz-Carretero A, Beau I, et al. Functional interaction between autophagy and ciliogenesis. *Nature* (2013) 502(7470):194–200. doi: 10.1038/nature12639
41. Nascimbeni AC, Giordano F, Dupont N, Grasso D, Vaccaro MI, Codogno P, et al. ER-plasma membrane contact sites contribute to autophagosome biogenesis by regulation of local PI3P synthesis. *EMBO J* (2017) 36 (14):2018–33. doi: 10.15252/embj.201797006
42. Axe EL, Walker SA, Manifava M, Chandra P, Roderick HL, Habermann A, et al. Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. *J Cell Biol* (2008) 182(4):685–701. doi: 10.1083/jcb.200803137
43. Yu L, Chen Y, Tooze SA. Autophagy pathway: Cellular and molecular mechanisms. *Autophagy* (2018) 14(2):207–15. doi: 10.1080/15548627.2017.1378838
44. Carlsson SR, Simonsen A. Membrane dynamics in autophagosome biogenesis. *J Cell Sci* (2015) 128(2):193–205. doi: 10.1242/jcs.141036
45. Mi N, Chen Y, Wang S, Chen M, Zhao M, Yang G, et al. CapZ regulates autophagosomal membrane shaping by promoting actin assembly inside the isolation membrane. *Nat Cell Biol* (2015) 17(9):1112–23. doi: 10.1038/ncb3215
46. Yang Z, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol* (2010) 22(2):124–31. doi: 10.1016/j.ceb.2009.11.014
47. Di Bartolomeo S, Corazzari M, Nazio F, Oliverio S, Lisi G, Antonioli M, et al. The dynamic interaction of AMBRA1 with the dynein motor complex regulates mammalian autophagy. *J Cell Biol* (2010) 191(1):155–68. doi: 10.1083/jcb.201002100
48. Ragusa MJ, Stanley RE, Hurley JH. Architecture of the Atg17 complex as a scaffold for autophagosome biogenesis. *Cell* (2012) 151(7):1501–12. doi: 10.1016/j.cell.2012.11.028
49. Wang J, Menon S, Yamasaki A, Chou HT, Walz T, Jiang Y, et al. Ypt1 recruits the Atg1 kinase to the preautophagosomal structure. *Proc Natl Acad Sci USA* (2013) 110(24):9800–5. doi: 10.1073/pnas.1302337110
50. Mathiowetz AJ, Baple E, Russo AJ, Coulter AM, Carrano E, Brown JD, et al. An Amish founder mutation disrupts a PI(3)P-WHAMM-Arp2/3 complex-driven autophagosomal remodeling pathway. *Mol Biol Cell* (2017) 28 (19):2492–507. doi: 10.1091/mbc.e17-01-0022
51. Coutts AS, La Thangue NB. Actin nucleation by WH2 domains at the autophagosome. *Nat Commun* (2015) 6:7888. doi: 10.1038/ncomms8888
52. Xia P, Wang S, Du Y, Zhao Z, Shi L, Sun L, et al. WASH inhibits autophagy through suppression of Beclin 1 ubiquitination. *EMBO J* (2013) 32 (20):2685–96. doi: 10.1038/emboj.2013.189
53. Sackmann E. The seventh Datta Lecture. Membrane bending energy concept of vesicle- and cell-shapes and shape-transitions. *FEBS Lett* (1994) 346(1):3–16. doi: 10.1016/0014-5793(94)00484-6
54. Chen Z, Rand RP. The influence of cholesterol on phospholipid membrane curvature and bending elasticity. *Biophys J* (1997) 73(1):267–76. doi: 10.1016/S0006-3495(97)78067-6
55. Stachowiak JC, Schmid EM, Ryan CJ, Ann HS, Sasaki DY, Sherman MB, et al. Membrane bending by protein-protein crowding. *Nat Cell Biol* (2012) 14(9):944–9. doi: 10.1038/ncb2561
56. Martens S, Nakamura S, Yoshimori T. Phospholipids in Autophagosome Formation and Fusion. *J Mol Biol* (2016) 428(24):4819–27 doi: 10.1016/j.jmb.2016.10.029
57. Staiano L, Zappa F. Hijacking intracellular membranes to feed autophagosomal growth. *FEBS Lett* (2019) 593(22):3120–34. doi: 10.1002/1873-3468.13637
58. Melia TJ, Lystad AH, Simonsen A. Autophagosome biogenesis: From membrane growth to closure. *J Cell Biol* (2020) 219(6):e202002085. doi: 10.1083/jcb.202002085
59. Chowdhury S, Otomo C, Leitner A, Ohashi K, Aebersold R, Lander GC, et al. Insights into autophagosome biogenesis from structural and biochemical analyses of the ATG2A-WIPI4 complex. *Proc Natl Acad Sci USA* (2018) 115 (42):E9792–E801. doi: 10.1073/pnas.1811874115
60. Jia R, Guardia CM, Pu J, Chen Y, Bonifacino JS. BORC coordinates encounter and fusion of lysosomes with autophagosomes. *Autophagy* (2017) 13(10):1648–63. doi: 10.1080/15548627.2017.1343768
61. Zhou C, Ma K, Gao R, Mu C, Chen L, Liu Q, et al. Regulation of mATG9 trafficking by Src- and ULK1-mediated phosphorylation in basal and starvation-induced autophagy. *Cell Res* (2017) 27(2):184–201. doi: 10.1038/cr.2016.146
62. Stenmark H. Rab GTPases as coordinators of vesicle traffic. *Nat Rev Mol Cell Biol* (2009) 10(8):513–25. doi: 10.1038/nrm2728
63. Longatti A, Lamb CA, Razi M, Yoshimura S, Barr FA, Tooze SA. TBC1D14 regulates autophagosome formation via Rab11- and ULK1-positive recycling endosomes. *J Cell Biol* (2012) 197(5):659–75. doi: 10.1083/jcb.201111079
64. Tang HW, Wang YB, Wang SL, Wu MH, Lin SY, Chen GC. Atg1-mediated myosin II activation regulates autophagosome formation during starvation-induced autophagy. *EMBO J* (2011) 30(4):636–51. doi: 10.1038/emboj.2010.338
65. Bialik S, Pietrokovski S, Kimchi A. Myosin drives autophagy in a pathway linking Atg1 to Atg9. *EMBO J* (2011) 30(4):629–30. doi: 10.1038/emboj.2011.8
66. Sawa-Makarska J, Baumann V, Coudevylle N, von Bulow S, Nogellova V, Abert C, et al. Reconstitution of autophagosome nucleation defines Atg9 vesicles as seeds for membrane formation. *Science* (2020) 369(6508): eaaz7714. doi: 10.1126/science.aaz7714
67. Monastyrska I, He C, Geng J, Hoppe AD, Li Z, Klionsky DJ. Arp2 links autophagic machinery with the actin cytoskeleton. *Mol Biol Cell* (2008) 19 (5):1962–75. doi: 10.1091/mbc.e07-09-0892
68. Tanida I, Ueno T, Kominami E. Human light chain 3/MAP1LC3B is cleaved at its carboxyl-terminal Met121 to expose Gly120 for lipidation and targeting to autophagosomal membranes. *J Biol Chem* (2004) 279 (46):47704–10. doi: 10.1074/jbc.M407016200
69. Parzych KR, Klionsky DJ. An overview of autophagy: morphology, mechanism, and regulation. *Antioxid Redox Signal* (2014) 20(3):460–73. doi: 10.1089/ars.2013.5371
70. Brier LW, Ge L, Stjepanovic G, Thelen AM, Hurley JH, Schekman R. Regulation of LC3 lipidation by the autophagy-specific class III phosphatidylinositol-3 kinase complex. *Mol Biol Cell* (2019) 30(9):1098–107. doi: 10.1091/mbc.E18-11-0743
71. Nath S, Dancourt J, Shteyn V, Puente G, Fong WM, Nag S, et al. Lipidation of the LC3/GABARAP family of autophagy proteins relies on a membrane-curvature-sensing domain in Atg3. *Nat Cell Biol* (2014) 16(5):415–24. doi: 10.1038/ncb2940
72. Dall'Armi C, Devereaux KA, Di Paolo G. The role of lipids in the control of autophagy. *Curr Biol* (2013) 23(1):R33–45. doi: 10.1016/j.cub.2012.10.041
73. Hailey DW, Rambold AS, Satpute-Krishnan P, Mitra K, Sougrat R, Kim PK, et al. Mitochondria supply membranes for autophagosome biogenesis during starvation. *Cell* (2010) 141(4):656–67. doi: 10.1016/j.cell.2010.04.009
74. McEwan DG, Dikic I. Not all autophagy membranes are created equal. *Cell* (2010) 141(4):564–6. doi: 10.1016/j.cell.2010.04.030
75. Birgisdottir AB, Lamark T, Johansen T. The LIR motif - crucial for selective autophagy. *J Cell Sci* (2013) 126(Pt 15):3237–47.doi: 10.1242/jcs.126128
76. Hu X, Mullins RD. LC3 and STRAP regulate actin filament assembly by JMY during autophagosome formation. *J Cell Biol* (2019) 218(1):251–66. doi: 10.1083/jcb.201802157
77. Hanna RA, Quinsay MN, Orogo AM, Giang K, Rikka S, Gustafsson AB. Microtubule-associated protein 1 light chain 3 (LC3) interacts with Bnip3 protein to selectively remove endoplasmic reticulum and mitochondria via autophagy. *J Biol Chem* (2012) 287(23):19094–104. doi: 10.1074/jbc.M111.322933
78. Fass E, Shvets E, Degani I, Hirschberg K, Elazar Z. Microtubules support production of starvation-induced autophagosomes but not their targeting

and fusion with lysosomes. *J Biol Chem* (2006) 281(47):36303–16. doi: 10.1074/jbc.M607031200
79. Weidberg H, Shvets E, Shpilka T, Shimron F, Shinder V, Elazar Z. LC3 and GATE-16/GABARAP subfamilies are both essential yet act differently in autophagosome biogenesis. *EMBO J* (2010) 29(11):1792–802. doi: 10.1038/emboj.2010.74
80. Ravikumar B, Sarkar S, Davies JE, Futter M, Garcia-Arencibia M, Green-Thompson ZW, et al. Regulation of mammalian autophagy in physiology and pathophysiology. *Physiol Rev* (2010) 90(4):1383–435. doi: 10.1152/physrev.00030.2009
81. Zhao YG, Zhang H. Autophagosome maturation: An epic journey from the ER to lysosomes. *J Cell Biol* (2019) 218(3):757–70. doi: 10.1083/jcb.201810099
82. Cebollero E, van der Vaart A, Zhao M, Rieter E, Klionsky DJ, Helms JB, et al. Phosphatidylinositol-3-phosphate clearance plays a key role in autophagosome completion. *Curr Biol* (2012) 22(17):1545–53. doi: 10.1016/j.cub.2012.06.029
83. Nakatogawa H, Ishii J, Asai E, Ohsumi Y. Atg4 recycles inappropriately lipidated Atg8 to promote autophagosome biogenesis. *Autophagy* (2012) 8 (2):177–86. doi: 10.4161/auto.8.2.18373
84. Lee YK, Lee JA. Role of the mammalian ATG8/LC3 family in autophagy: differential and compensatory roles in the spatiotemporal regulation of autophagy. *BMB Rep* (2016) 49(8):424–30. doi: 10.5483/BMBRep.2016.49.8.081
85. Knorr RL, Lipowsky R, Dimova R. Autophagosome closure requires membrane scission. *Autophagy* (2015) 11(11):2134–7. doi: 10.1080/15548627.2015.1091552
86. Li L, Tong M, Fu Y, Chen F, Zhang S, Chen H, et al. Lipids and membrane-associated proteins in autophagy. *Protein Cell* (2020) 1–25. doi: 10.1007/s13238-020-00793-9
87. Yu S, Melia TJ. The coordination of membrane fission and fusion at the end of autophagosome maturation. *Curr Opin Cell Biol* (2017) 47:92–8. doi: 10.1016/j.ceb.2017.03.010
88. Takahashi Y, Liang X, Hattori T, Tang Z, He H, Chen H, et al. VPS37A directs ESCRT recruitment for phagophore closure. *J Cell Biol* (2019) 218 (10):3336–54. doi: 10.1083/jcb.201902170
89. Christ L, Raiborg C, Wenzel EM, Campsteijn C, Stenmark H. Cellular Functions and Molecular Mechanisms of the ESCRT Membrane-Scission Machinery. *Trends Biochem Sci* (2017) 42(1):42–56. doi: 10.1016/j.tibs.2016.08.016
90. Schoneberg J, Lee IH, Iwasa JH, Hurley JH. Reverse-topology membrane scission by the ESCRT proteins. *Nat Rev Mol Cell Biol* (2017) 18(1):5–17. doi: 10.1038/nrm.2016.121
91. Chiaruttini N, Redondo-Morata L, Colom A, Humbert F, Lenz M, Scheuring S, et al. Relaxation of Loaded ESCRT-III Spiral Springs Drives Membrane Deformation. *Cell* (2015) 163(4):866–79. doi: 10.1016/j.cell.2015.10.017
92. Adell MA, Vogel GF, Pakdel M, Muller M, Lindner H, Hess MW, et al. Coordinated binding of Vps4 to ESCRT-III drives membrane neck constriction during MVB vesicle formation. *J Cell Biol* (2014) 205(1):33–49. doi: 10.1083/jcb.201310114
93. Corona AK, Jackson WT. Finding the Middle Ground for Autophagic Fusion Requirements. *Trends Cell Biol* (2018) 28(11):869–81. doi: 10.1016/j.tcb.2018.07.001
94. Trivedi PC, Bartlett JJ, Pulinilkunnil T. Lysosomal Biology and Function: Modern View of Cellular Debris Bin. *Cells* (2020) 9(5):1131. doi: 10.3390/cells9051131
95. Itakura E, Kishi-Itakura C, Mizushima N. The hairpin-type tail-anchored SNARE syntaxin 17 targets to autophagosomes for fusion with endosomes/lysosomes. *Cell* (2012) 151(6):1256–69. doi: 10.1016/j.cell.2012.11.001
96. Diao J, Liu R, Rong Y, Zhao M, Zhang J, Lai Y, et al. ATG14 promotes membrane tethering and fusion of autophagosomes to endolysosomes. *Nature* (2015) 520(7548):563–6. doi: 10.1038/nature14147
97. Guerra F, Bucci C. Multiple Roles of the Small GTPase Rab7. *Cells* (2016) 5 (3):34. doi: 10.3390/cells5030034
98. Miao G, Zhang Y, Chen D, Zhang H. The ER-Localized Transmembrane Protein TMEM39A/SUSR2 Regulates Autophagy by Controlling the Trafficking of the PtdIns(4)P Phosphatase SAC1. *Mol Cell* (2020) 77 (3):618–32.e5. doi: 10.1016/j.molcel.2019.10.035
99. Manil-Segalen M, Lefebvre C, Jenzer C, Trichet M, Boulogne C, Satiat-Jeunemaitre B, et al. The C. elegans LC3 acts downstream of GABARAP to degrade autophagosomes by interacting with the HOPS subunit VPS39. *Dev Cell* (2014) 28(1):43–55. doi: 10.1016/j.devcel.2013.11.022
100. McEwan DG, Popovic D, Gubas A, Terawaki S, Suzuki H, Stadel D, et al. PLEKHM1 regulates autophagosome-lysosome fusion through HOPS complex and LC3/GABARAP proteins. *Mol Cell* (2015) 57(1):39–54. doi: 10.1016/j.molcel.2014.11.006
101. Jiang P, Nishimura T, Sakamaki Y, Itakura E, Hatta T, Natsume T, et al. The HOPS complex mediates autophagosome-lysosome fusion through interaction with syntaxin 17. *Mol Biol Cell* (2014) 25(8):1327–37. doi: 10.1091/mbc.e13-08-0447
102. Chen D, Fan W, Lu Y, Ding X, Chen S, Zhong Q. A mammalian autophagosome maturation mechanism mediated by TECPR1 and the Atg12-Atg5 conjugate. *Mol Cell* (2012) 45(5):629–41. doi: 10.1016/j.molcel.2011.12.036
103. Wetzel L, Blanchard S, Rama S, Beier V, Kaufmann A, Wollert T. TECPR1 promotes aggrephagy by direct recruitment of LC3C autophagosomes to lysosomes. *Nat Commun* (2020) 11(1):2993. doi: 10.1038/s41467-020-16689-5
104. Zhang Y, Jiang X, Deng Q, Gao Z, Tang X, Fu R, et al. Downregulation of MYO1C mediated by cepharanthine inhibits autophagosome-lysosome fusion through blockade of the F-actin network. *J Exp Clin Cancer Res* (2019) 38(1):457. doi: 10.1186/s13046-019-1449-8
105. Kim GH, Dayam RM, Prashar A, Terebiznik M, Botelho RJ. PIKfyve inhibition interferes with phagosome and endosome maturation in macrophages. *Traffic* (2014) 15(10):1143–63. doi: 10.1111/tra.12199
106. Kruppa AJ, Kendrick-Jones J, Buss F. Myosins, Actin and Autophagy. *Traffic* (2016) 17(8):878–90. doi: 10.1111/tra.12410
107. Bose A, Robida S, Furcinitti PS, Chawla A, Fogarty K, Corvera S, et al. Unconventional myosin Myo1c promotes membrane fusion in a regulated exocytic pathway. *Mol Cell Biol* (2004) 24(12):5447–58. doi: 10.1128/MCB.24.12.5447-5458.2004
108. Dai A, Yu L, Wang HW. WHAMM initiates autolysosome tubulation by promoting actin polymerization on autolysosomes. *Nat Commun* (2019) 10 (1):3699. doi: 10.1038/s41467-019-11694-9
109. Freeman SA, Grinstein S. Resolution of macropinosomes, phagosomes and autolysosomes: Osmotically driven shrinkage enables tubulation and vesiculation. *Traffic* (2018) 19(12):965–74. doi: 10.1111/tra.12614
110. McMahon HT, Gallop JL. Membrane curvature and mechanisms of dynamic cell membrane remodelling. *Nature* (2005) 438(7068):590–6. doi: 10.1038/nature04396
111. Sainio A, Jarvelainen H. Extracellular matrix macromolecules: potential tools and targets in cancer gene therapy. *Mol Cell Ther* (2014) 2:14. doi: 10.1186/2052-8426-2-14
112. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell* (2006) 126(4):677–89. doi: 10.1016/j.cell.2006.06.044
113. Vogel V, Sheetz M. Local force and geometry sensing regulate cell functions. *Nat Rev Mol Cell Biol* (2006) 7(4):265–75. doi: 10.1038/nrm1890
114. Doss BL, Pan M, Gupta M, Grenci G, Mege RM, Lim CT, et al. Cell response to substrate rigidity is regulated by active and passive cytoskeletal stress. *Proc Natl Acad Sci USA* (2020) 117(23):12817–25. doi: 10.1073/pnas.1917555117
115. Vedula SR, Leong MC, Lai TL, Hersen P, Kabla AJ, Lim CT, et al. Emerging modes of collective cell migration induced by geometrical constraints. *Proc Natl Acad Sci USA* (2012) 109(32):12974–9. doi: 10.1073/pnas.1119313109
116. Ravasio A, Cheddadi I, Chen T, Pereira T, Ong HT, Bertocchi C, et al. Gap geometry dictates epithelial closure efficiency. *Nat Commun* (2015) 6:7683. doi: 10.1038/ncomms8683
117. Baumgarten C, Feher J. Osmosis and Regulation of Cell Volume. In: Sperelakis N, editor. *Cell Physiology Source Book*, *4th ed.* Ohio, U.S.A.: Academic Press (2012). p. 261–301.
118. Dietl P, Frick M, Mair N, Bertocchi C, Haller T. Pulmonary consequences of a deep breath revisited. *Biol Neonate* (2004) 85(4):299–304. doi: 10.1159/000078176
119. Ravasio A, Hobi N, Bertocchi C, Jesacher A, Dietl P, Haller T. Interfacial sensing by alveolar type II cells: a new concept in lung physiology? *Am J Physiol Cell Physiol* (2011) 300(6):C1456–65. doi: 10.1152/ajpcell.00427.2010
120. Shivashankar GV, Sheetz M, Matsudaira P. Mechanobiology. *Integr Biol (Camb)* (2015) 7(10):1091–2. doi: 10.1039/C5IB90040A

121. Jennings P, Bertocchi C, Frick M, Haller T, Pfaller W, Dietl P. Ca^{2+} Induced Surfactant Secretion in Alveolar Type II Cultures Isolated from the H-2Kb-tsA58 Transgenic Mouse. *Cell Physiol Biochem* (2005) 15(1-4):159–66. doi: 10.1159/000083648
122. Fletcher DA, Mullins RD. Cell mechanics and the cytoskeleton. *Nature* (2010) 463(7280):485–92. doi: 10.1038/nature08908
123. Chen Y, Ju L, Rushdi M, Ge C, Zhu C. Receptor-mediated cell mechanosensing. *Mol Biol Cell* (2017) 28(23):3134–55. doi: 10.1091/mbc.e17-04-0228
124. Wolfenson H, Yang B, Sheetz MP. Steps in Mechanotransduction Pathways that Control Cell Morphology. *Annu Rev Physiol* (2019) 81:585–605. doi: 10.1146/annurev-physiol-021317-121245
125. Han Y, Liu C, Zhang D, Men H, Huo L, Geng Q, et al. Mechanosensitive ion channel Piezo1 promotes prostate cancer development through the activation of the Akt/mTOR pathway and acceleration of cell cycle. *Int J Oncol* (2019) 55(3):629–44. doi: 10.3892/ijo.2019.4839
126. Guo Y, Steele HE, Li BY, Na S. Fluid flow-induced activation of subcellular AMPK and its interaction with FAK and Src. *Arch Biochem Biophys* (2020) 679:108208. doi: 10.1016/j.abb.2019.108208
127. Dower CM, Wills CA, Frisch SM, Wang HG. Mechanisms and context underlying the role of autophagy in cancer metastasis. *Autophagy* (2018) 14 (7):1110–28. doi: 10.1080/15548627.2018.1450020
128. King JS. Mechanical stress meets autophagy: potential implications for physiology and pathology. *Trends Mol Med* (2012) 18(10):583–8. doi: 10.1016/j.molmed.2012.08.002
129. Das J, Agarwal T, Chakraborty S, Maiti TK. Compressive stress-induced autophagy promotes invasion of HeLa cells by facilitating protein turnover in vitro. *Exp Cell Res* (2019) 381(2):201–7. doi: 10.1016/j.yexcr.2019.04.037
130. Das J, Maji S, Agarwal T, Chakraborty S, Maiti TK. Hemodynamic shear stress induces protective autophagy in HeLa cells through lipid raft-mediated mechanotransduction. *Clin Exp Metastasis* (2018) 35(3):135–48. doi: 10.1007/s10585-018-9887-9
131. Vogel V. Unraveling the Mechanobiology of Extracellular Matrix. *Annu Rev Physiol* (2018) 80:353–87. doi: 10.1146/annurev-physiol-021317-121312
132. Jiang G, Huang AH, Cai Y, Tanase M, Sheetz MP. Rigidity sensing at the leading edge through alphavbeta3 integrins and RPTPalpha. *Biophys J* (2006) 90(5):1804–9. doi: 10.1529/biophysj.105.072462
133. Ross TD, Coon BG, Yun S, Baeyens N, Tanaka K, Ouyang M, et al. Integrins in mechanotransduction. *Curr Opin Cell Biol* (2013) 25(5):613–8. doi: 10.1016/j.ceb.2013.05.006
134. Wozniak MA, Modzelewska K, Kwong L, Keely PJ. Focal adhesion regulation of cell behavior. *Biochim Biophys Acta* (2004) 1692(2-3):103–19. doi: 10.1016/j.bbamcr.2004.04.007
135. Kim M, Carman CV, Springer TA. Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. *Science* (2003) 301(5640):1720–5. doi: 10.1126/science.1084174
136. Luo BH, Carman CV, Springer TA. Structural basis of integrin regulation and signaling. *Annu Rev Immunol* (2007) 25:619–47. doi: 10.1146/annurev.immunol.25.022106.141618
137. Caswell PT, Vadrevu S, Norman JC. Integrins: masters and slaves of endocytic transport. *Nat Rev Mol Cell Biol* (2009) 10(12):843–53. doi: 10.1038/nrm2799
138. Seetharaman S, Etienne-Manneville S. Integrin diversity brings specificity in mechanotransduction. *Biol Cell* (2018) 110(3):49–64. doi: 10.1111/boc.201700060
139. Lock R, Debnath J. Extracellular matrix regulation of autophagy. *Curr Opin Cell Biol* (2008) 20(5):583–8. doi: 10.1016/j.ceb.2008.05.002
140. Vlahakis A, Debnath J. The Interconnections between Autophagy and Integrin-Mediated Cell Adhesion. *J Mol Biol* (2017) 429(4):515–30. doi: 10.1016/j.jmb.2016.11.027
141. Anlas AA, Nelson CM. Soft Microenvironments Induce Chemoresistance by Increasing Autophagy Downstream of Integrin-Linked Kinase. *Cancer Res* (2020) 80(19):4103–13. doi: 10.1158/0008-5472.CAN-19-4021
142. Gubbiotti MA, Iozzo RV. Proteoglycans regulate autophagy via outside-in signaling: an emerging new concept. *Matrix Biol* (2015) 48:6–13. doi: 10.1016/j.matbio.2015.10.002
143. Nguyen TM, Subramanian IV, Kelekar A, Ramakrishnan S. Kringle 5 of human plasminogen, an angiogenesis inhibitor, induces both autophagy and apoptotic death in endothelial cells. *Blood* (2007) 109(11):4793–802. doi: 10.1182/blood-2006-11-059352
144. Nguyen TM, Subramanian IV, Xiao X, Ghosh G, Nguyen P, Kelekar A, et al. Endostatin induces autophagy in endothelial cells by modulating Beclin 1 and beta-catenin levels. *J Cell Mol Med* (2009) 13(9B):3687–98. doi: 10.1111/j.1582-4934.2009.00722.x
145. Castagnaro S, Chrisam M, Cescon M, Braghetta P, Grumati P, Bonaldo P. Extracellular Collagen VI Has Prosurvival and Autophagy Instructive Properties in Mouse Fibroblasts. *Front Physiol* (2018) 9:1129. doi: 10.3389/fphys.2018.01129
146. Carmignac V, Svensson M, Korner Z, Elowsson L, Matsumura C, Gawlik KI, et al. Autophagy is increased in laminin alpha2 chain-deficient muscle and its inhibition improves muscle morphology in a mouse model of MDC1A. *Hum Mol Genet* (2011) 20(24):4891–902. doi: 10.1093/hmg/ddr427
147. Neill T, Schaefer L, Iozzo RV. Instructive roles of extracellular matrix on autophagy. *Am J Pathol* (2014) 184(8):2146–53. doi: 10.1016/j.ajpath.2014.05.010
148. Neill T, Schaefer L, Iozzo RV. Decorin: a guardian from the matrix. *Am J Pathol* (2012) 181(2):380–7. doi: 10.1016/j.ajpath.2012.04.029
149. Sharifi MN, Mowers EE, Drake LE, Collier C, Chen H, Zamora M, et al. Autophagy Promotes Focal Adhesion Disassembly and Cell Motility of Metastatic Tumor Cells through the Direct Interaction of Paxillin with LC3. *Cell Rep* (2016) 15(8):1660–72. doi: 10.1016/j.celrep.2016.04.065
150. Angulo-Urarte A, van der Wal T, Huveneers S. Cell-cell junctions as sensors and transducers of mechanical forces. *Biochim Biophys Acta Biomembr* (2020) 1862(9):183316. doi: 10.1016/j.bbamem.2020.183316
151. Zaidel-Bar R. Cadherin adhesome at a glance. *J Cell Sci* (2013) 126(Pt 2):373–8. doi: 10.1242/jcs.111559
152. Han MK, de Rooij J. Resolving the cadherin-F-actin connection. *Nat Cell Biol* (2016) 19(1):14–6. doi: 10.1038/ncb3457
153. Bertocchi C, Wang Y, Ravasio A, Hara Y, Wu Y, Sailov T, et al. Nanoscale architecture of cadherin-based cell adhesions. *Nat Cell Biol* (2017) 19(1):28–37. doi: 10.1038/ncb3456
154. Nighot P, Ma T. Role of autophagy in the regulation of epithelial cell junctions. *Tissue Barriers* (2016) 4(3):e1171284. doi: 10.1080/21688370.2016.1171284
155. Damiano V, Spessotto P, Vanin G, Perin T, Maestro R, Santarosa M. The Autophagy Machinery Contributes to E-cadherin Turnover in Breast Cancer. *Front Cell Dev Biol* (2020) 8:545. doi: 10.3389/fcell.2020.00545
156. Hu CA, Hou Y, Yi D, Qiu Y, Wu G, Kong X, et al. Autophagy and tight junction proteins in the intestine and intestinal diseases. *Anim Nutr* (2015) 1 (3):123–7. doi: 10.1016/j.aninu.2015.08.014
157. Bays JL, Campbell HK, Heidema C, Sebbagh M, DeMali KA. Linking E-cadherin mechanotransduction to cell metabolism through force-mediated activation of AMPK. *Nat Cell Biol* (2017) 19(6):724–31. doi: 10.1038/ncb3537
158. Dupont S, Morsut L, Aragona M, Enzo E, Giulitti S, Cordenonsi M, et al. Role of YAP/TAZ in mechanotransduction. *Nature* (2011) 474(7350):179–83. doi: 10.1038/nature10137
159. Zhao B, Ye X, Yu J, Li L, Li W, Li S, et al. TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev* (2008) 22(14):1962–71. doi: 10.1101/gad.1664408
160. Zhou Y, Huang T, Cheng AS, Yu J, Kang W, To KF. The TEAD Family and Its Oncogenic Role in Promoting Tumorigenesis. *Int J Mol Sci* (2016) 17 (1):138. doi: 10.3390/ijms17010138
161. Wada K, Itoga K, Okano T, Yonemura S, Sasaki H. Hippo pathway regulation by cell morphology and stress fibers. *Development* (2011) 138 (18):3907–14. doi: 10.1242/dev.070987
162. Aragona M, Panciera T, Manfrin A, Giulitti S, Michielin F, Elvassore N, et al. A mechanical checkpoint controls multicellular growth through YAP/TAZ regulation by actin-processing factors. *Cell* (2013) 154(5):1047–59. doi: 10.1016/j.cell.2013.07.042
163. Totaro A, Zhuang Q, Panciera T, Battilana G, Azzolin L, Brumana G, et al. Cell phenotypic plasticity requires autophagic flux driven by YAP/TAZ mechanotransduction. *Proc Natl Acad Sci USA* (2019) 116(36):17848–57. doi: 10.1073/pnas.1908228116
164. Carroll B, Mohd-Naim N, Maximiano F, Frasa MA, McCormack J, Finelli M, et al. The TBC/RabGAP Armus coordinates Rac1 and Rab7 functions during autophagy. *Dev Cell* (2013) 25(1):15–28. doi: 10.1016/j.devcel.2013.03.005

165. Liang N, Zhang C, Dill P, Panasyuk G, Pion D, Koka V, et al. Regulation of YAP by mTOR and autophagy reveals a therapeutic target of tuberous sclerosis complex. *J Exp Med* (2014) 211(11):2249–63. doi: 10.1084/jem.20140341
166. Pocaterra A, Romani P, Dupont S. YAP/TAZ functions and their regulation at a glance. *J Cell Sci* (2020) 133(2):jcs230425. doi: 10.1242/jcs.230425
167. Kondratskyi A, Kondratska K, Skryma R, Klionsky DJ, Prevarskaya N. Ion channels in the regulation of autophagy. *Autophagy* (2018) 14(1):3–21. doi: 10.1080/15548627.2017.1384887
168. Moroni M, Servin-Vences MR, Fleischer R, Sanchez-Carranza O, Lewin GR. Voltage gating of mechanosensitive PIEZO channels. *Nat Commun* (2018) 9(1):1096. doi: 10.1038/s41467-018-03502-7
169. Liedtke WB. *TRP Ion Channel Function in Sensory Transduction and Cellular Signaling Cascades*. Durham, North Carolina and Stanford, California, U.S.A.: CRC Press/Taylor & Francis (2006).
170. Ayad NME, Kaushik S, Weaver VM. Tissue mechanics, an important regulator of development and disease. *Philos Trans R Soc Lond B Biol Sci* (2019) 374(1779):20180215. doi: 10.1098/rstb.2018.0215
171. Le Roux AL, Quiroga X, Walani N, Arroyo M, Roca-Cusachs P. The plasma membrane as a mechanochemical transducer. *Philos Trans R Soc Lond B Biol Sci* (2019) 374(1779):20180221. doi: 10.1098/rstb.2018.0221
172. Yan Z, Su G, Gao W, He J, Shen Y, Zeng Y, et al. Fluid shear stress induces cell migration and invasion via activating autophagy in HepG2 cells. *Cell Adh Migr* (2019) 13(1):152–63. doi: 10.1080/19336918.2019.1568141
173. King JS, Veltman DM, Insall RH. The induction of autophagy by mechanical stress. *Autophagy* (2011) 7(12):1490–9. doi: 10.4161/auto.7.12.17924
174. Peter BJ, Kent HM, Mills IG, Vallis Y, Butler PJ, Evans PR, et al. BAR domains as sensors of membrane curvature: the amphiphysin BAR structure. *Science* (2004) 303(5657):495–9. doi: 10.1126/science.1092586
175. Glogauer M, Arora P, Chou D, Janmey PA, Downey GP, McCulloch CA. The role of actin-binding protein 280 in integrin-dependent mechanoprotection. *J Biol Chem* (1998) 273(3):1689–98. doi: 10.1074/jbc.273.3.1689
176. Razinia Z, Makela T, Ylanne J, Calderwood DA. Filamins in mechanosensing and signaling. *Annu Rev Biophys* (2012) 41:227–46. doi: 10.1146/annurev-biophys-050511-102252
177. Jackson WM, Jaasma MJ, Tang RY, Keaveny TM. Mechanical loading by fluid shear is sufficient to alter the cytoskeletal composition of osteoblastic cells. *Am J Physiol Cell Physiol* (2008) 295(4):C1007–15. doi: 10.1152/ajpcell.00509.2007
178. Gehler S, Baldassarre M, Lad Y, Leight JL, Wozniak MA, Riching KM, et al. Filamin A-beta1 integrin complex tunes epithelial cell response to matrix tension. *Mol Biol Cell* (2009) 20(14):3224–38. doi: 10.1091/mbc.e08-12-1186
179. Ulbricht A, Eppler FJ, Tapia VE, van der Ven PF, Hampe N, Hersch N, et al. Cellular mechanotransduction relies on tension-induced and chaperone-assisted autophagy. *Curr Biol* (2013) 23(5):430–5. doi: 10.1016/j.cub.2013.01.064
180. Satir P, Pedersen LB, Christensen ST. The primary cilium at a glance. *J Cell Sci* (2010) 123(Pt 4):499–503. doi: 10.1242/jcs.050377
181. Praetorius HA. The primary cilium as sensor of fluid flow: new building blocks to the model. A review in the theme: cell signaling: proteins, pathways and mechanisms. *Am J Physiol Cell Physiol* (2015) 308(3):C198–208. doi: 10.1152/ajpcell.00336.2014
182. Orhon I, Dupont N, Codogno P. Primary cilium and autophagy: The avengers of cell-size regulation. *Autophagy* (2016) 12(11):2258–9. doi: 10.1080/15548627.2016.1212790
183. Praetorius HA, Spring KR. Bending the MDCK cell primary cilium increases intracellular calcium. *J Membr Biol* (2001) 184(1):71–9. doi: 10.1007/s00232-001-0075-4
184. Orhon I, Dupont N, Zaidan M, Boitez V, Burtin M, Schmitt A, et al. Primary-cilium-dependent autophagy controls epithelial cell volume in response to fluid flow. *Nat Cell Biol* (2016) 18(6):657–67. doi: 10.1038/ncb3360
185. Zemirli N, Boukhalfa A, Dupont N, Botti J, Codogno P, Morel E. The primary cilium protein folliculin is part of the autophagy signaling pathway to regulate epithelial cell size in response to fluid flow. *Cell Stress* (2019) 3(3):100–9. doi: 10.15698/cst2019.03.180
186. Boehlke C, Kotsis F, Patel V, Braeg S, Voelker H, Bredt S, et al. Primary cilia regulate mTORC1 activity and cell size through Lkb1. *Nat Cell Biol* (2010) 12(11):1115–22. doi: 10.1038/ncb2117
187. Boukhalfa A, Nascimbeni AC, Dupont N, Codogno P, Morel E. Primary cilium-dependent autophagy drafts PIK3C2A to generate PtdIns3P in response to shear stress. *Autophagy* (2020) 16(6):1143–4. doi: 10.1080/15548627.2020.1732687
188. Boukhalfa A, Nascimbeni AC, Ramel D, Dupont N, Hirsch E, Gayral S, et al. PI3KC2alpha-dependent and VPS34-independent generation of PI3P controls primary cilium-mediated autophagy in response to shear stress. *Nat Commun* (2020) 11(1):294. doi: 10.1038/s41467-019-14086-1
189. Franco I, Gulluni F, Campa CC, Costa C, Margaria JP, Ciraolo E, et al. PI3K class II alpha controls spatially restricted endosomal PtdIns3P and Rab11 activation to promote primary cilium function. *Dev Cell* (2014) 28(6):647–58. doi: 10.1016/j.devcel.2014.01.022
190. Campa CC, Margaria JP, Derle A, Del Giudice M, De Santis MC, Gozzelino L, et al. Rab11 activity and PtdIns(3)P turnover removes recycling cargo from endosomes. *Nat Chem Biol* (2018) 14(8):801–10. doi: 10.1038/s41589-018-0086-4
191. Puri C, Vicinanza M, Ashkenazi A, Gratian MJ, Zhang Q, Bento CF, et al. The RAB11A-Positive Compartment Is a Primary Platform for Autophagosome Assembly Mediated by WIPI2 Recognition of PI3P-RAB11A. *Dev Cell* (2018) 45(1):114–31.e8. doi: 10.1016/j.devcel.2018.03.008
192. Vicinanza M, Puri C, Rubinsztein DC. Coincidence detection of RAB11A and PI(3)P by WIPI2 directs autophagosome formation. *Oncotarget* (2019) 10(27):2579–80. doi: 10.18632/oncotarget.26829
193. Pierce NW, Nachury MV. Cilia grow by taking a bite out of the cell. *Dev Cell* (2013) 27(2):126–7. doi: 10.1016/j.devcel.2013.10.013
194. Tang Z, Lin MG, Stowe TR, Chen S, Zhu M, Stearns T, et al. Autophagy promotes primary ciliogenesis by removing OFD1 from centriolar satellites. *Nature* (2013) 502(7470):254–7. doi: 10.1038/nature12606
195. Kumar S, Weaver VM. Mechanics, malignancy, and metastasis: the force journey of a tumor cell. *Cancer Metastasis Rev* (2009) 28(1-2):113–27. doi: 10.1007/s10555-008-9173-4
196. Ravasio A, Le AP, Saw TB, Tarle V, Ong HT, Bertocchi C, et al. Regulation of epithelial cell organization by tuning cell-substrate adhesion. *Integr Biol (Camb)* (2015) 7(10):1228–41. doi: 10.1039/C5IB00196J
197. Sharma A, Seow JJW, Dutertre CA, Pai R, Bleriot C, Mishra A, et al. Onco-fetal Reprogramming of Endothelial Cells Drives Immunosuppressive Macrophages in Hepatocellular Carcinoma. *Cell* (2020) 183(2):377–94 e21. doi: 10.1016/j.cell.2020.08.040
198. White E, DiPaola RS. The double-edged sword of autophagy modulation in cancer. *Clin Cancer Res* (2009) 15(17):5308–16. doi: 10.1158/1078-0432.CCR-07-5023
199. Bhutia SK, Mukhopadhyay S, Sinha N, Das DN, Panda PK, Patra SK, et al. Autophagy: cancer's friend or foe? *Adv Cancer Res* (2013) 118:61–95. doi: 10.1016/B978-0-12-407173-5.00003-0
200. Morselli E, Galluzzi L, Kepp O, Vicencio JM, Criollo A, Maiuri MC, et al. Anti- and pro-tumor functions of autophagy. *Biochim Biophys Acta* (2009) 1793(9):1524–32. doi: 10.1016/j.bbamcr.2009.01.006
201. Li X, Wang J. Mechanical tumor microenvironment and transduction: cytoskeleton mediates cancer cell invasion and metastasis. *Int J Biol Sci* (2020) 16(12):2014–28. doi: 10.7150/ijbs.44943
202. Panda PK, Mukhopadhyay S, Das DN, Sinha N, Naik PP, Bhutia SK. Mechanism of autophagic regulation in carcinogenesis and cancer therapeutics. *Semin Cell Dev Biol* (2015) 39:43–55. doi: 10.1016/j.semcdb.2015.02.013
203. Jain RK, Martin JD, Stylianopoulos T. The role of mechanical forces in tumor growth and therapy. *Annu Rev BioMed Eng* (2014) 16:321–46. doi: 10.1146/annurev-bioeng-071813-105259
204. Nia HT, Liu H, Seano G, Datta M, Jones D, Rahbari N, et al. Solid stress and elastic energy as measures of tumour mechanopathology. *Nat BioMed Eng* (2016) 1:0004. doi: 10.1038/s41551-016-0004
205. Shieh AC, Swartz MA. Regulation of tumor invasion by interstitial fluid flow. *Phys Biol* (2011) 8(1):015012. doi: 10.1088/1478-3975/8/1/015012
206. Hyler AR, Baudoin NC, Brown MS, Stremler MA, Cimini D, Davalos RV, et al. Fluid shear stress impacts ovarian cancer cell viability, subcellular organization, and promotes genomic instability. *PloS One* (2018) 13(3):e0194170. doi: 10.1371/journal.pone.0194170
207. Stylianopoulos T. The Solid Mechanics of Cancer and Strategies for Improved Therapy. *J Biomech Eng* (2017) 139(2):021004. doi: 10.1115/1.4034991

208. Stylianopoulos T, Martin JD, Snuderl M, Mpekris F, Jain SR, Jain RK. Coevolution of solid stress and interstitial fluid pressure in tumors during progression: implications for vascular collapse. *Cancer Res* (2013) 73 (13):3833–41. doi: 10.1158/0008-5472.CAN-12-4521
209. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol* (2014) 15(3):178–96. doi: 10.1038/nrm3758
210. Wirtz D, Konstantopoulos K, Searson PC. The physics of cancer: the role of physical interactions and mechanical forces in metastasis. *Nat Rev Cancer* (2011) 11(7):512–22. doi: 10.1038/nrc3080
211. Xie L, Law BK, Chytil AM, Brown KA, Aakre ME, Moses HL. Activation of the Erk pathway is required for TGF-beta1-induced EMT in vitro. *Neoplasia* (2004) 6(5):603–10. doi: 10.1593/neo.04241
212. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* (2003) 425(6958):577–84. doi: 10.1038/nature02006
213. Ridley AJ. Life at the leading edge. *Cell* (2011) 145(7):1012–22. doi: 10.1016/j.cell.2011.06.010
214. Gilkes DM, Semenza GL, Wirtz D. Hypoxia and the extracellular matrix: drivers of tumour metastasis. *Nat Rev Cancer* (2014) 14(6):430–9. doi: 10.1038/nrc3726
215. Stylianou A, Gkretsi V, Stylianopoulos T. Transforming growth factor-beta modulates pancreatic cancer associated fibroblasts cell shape, stiffness and invasion. *Biochim Biophys Acta Gen Subj* (2018) 1862(7):1537–46. doi: 10.1016/j.bbagen.2018.02.009
216. Gaggioli C, Hooper S, Hidalgo-Carcedo C, Grosse R, Marshall JF, Harrington K, et al. Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. *Nat Cell Biol* (2007) 9(12):1392–400. doi: 10.1038/ncb1658
217. Garcia-Palmero I, Torres S, Bartolome RA, Pelaez-Garcia A, Larriba MJ, Lopez-Lucendo M, et al. Twist1-induced activation of human fibroblasts promotes matrix stiffness by upregulating palladin and collagen alpha1(VI). *Oncogene* (2016) 35(40):5224–36. doi: 10.1038/onc.2016.57
218. Najafi M, Farhood B, Mortezaee K. Extracellular matrix (ECM) stiffness and degradation as cancer drivers. *J Cell Biochem* (2019) 120(3):2782–90. doi: 10.1002/jcb.27681
219. Yu H, Mouw JK, Weaver VM. Forcing form and function: biomechanical regulation of tumor evolution. *Trends Cell Biol* (2011) 21(1):47–56. doi: 10.1016/j.tcb.2010.08.015
220. Plaks V, Koopman CD, Werb Z. Cancer. Circulating tumor cells. *Science* (2013) 341(6151):1186–8. doi: 10.1126/science.1235226
221. Massague J, Obenauf AC. Metastatic colonization by circulating tumour cells. *Nature* (2016) 529(7586):298–306. doi: 10.1038/nature17038
222. Strilic B, Offermanns S. Intravascular Survival and Extravasation of Tumor Cells. *Cancer Cell* (2017) 32(3):282–93. doi: 10.1016/j.ccell.2017.07.001
223. Fan R, Emery T, Zhang Y, Xia Y, Sun J, Wan J. Circulatory shear flow alters the viability and proliferation of circulating colon cancer cells. *Sci Rep* (2016) 6:27073. doi: 10.1038/srep27073
224. Avvisato CL, Yang X, Shah S, Hoxter B, Li W, Gaynor R, et al. Mechanical force modulates global gene expression and beta-catenin signaling in colon cancer cells. *J Cell Sci* (2007) 120(Pt 15):2672–82. doi: 10.1242/jcs.03476
225. Mitchell MJ, King MR. Fluid Shear Stress Sensitizes Cancer Cells to Receptor-Mediated Apoptosis via Trimeric Death Receptors. *New J Phys* (2013) 15:015008. doi: 10.1088/1367-2630/15/1/015008
226. Ma S, Fu A, Chiew GG, Luo KQ. Hemodynamic shear stress stimulates migration and extravasation of tumor cells by elevating cellular oxidative level. *Cancer Lett* (2017) 388:239–48. doi: 10.1016/j.canlet.2016.12.001
227. Xiong N, Li S, Tang K, Bai H, Peng Y, Yang H, et al. Involvement of caveolin-1 in low shear stress-induced breast cancer cell motility and adhesion: Roles of FAK/Src and ROCK/p-MLC pathways. *Biochim Biophys Acta Mol Cell Res* (2017) 1864(1):12–22. doi: 10.1016/j.bbamcr.2016.10.013
228. Wang X, Zhang Y, Feng T, Su G, He J, Gao W, et al. Fluid Shear Stress Promotes Autophagy in Hepatocellular Carcinoma Cells. *Int J Biol Sci* (2018) 14(10):1277–90. doi: 10.7150/ijbs.27055
229. Regmi S, Fu A, Luo KQ. High Shear Stresses under Exercise Condition Destroy Circulating Tumor Cells in a Microfluidic System. *Sci Rep* (2017) 7:39975. doi: 10.1038/srep39975
230. Lien SC, Chang SF, Lee PL, Wei SY, Chang MD, Chang JY, et al. Mechanical regulation of cancer cell apoptosis and autophagy: roles of bone morphogenetic protein receptor, Smad1/5, and p38 MAPK. *Biochim Biophys Acta* (2013) 1833(12):3124–33. doi: 10.1016/j.bbamcr.2013.08.023
231. Chaudhuri O, Koshy ST, Branco da Cunha C, Shin JW, Verbeke CS, Allison KH, et al. Extracellular matrix stiffness and composition jointly regulate the induction of malignant phenotypes in mammary epithelium. *Nat Mater* (2014) 13(10):970–8. doi: 10.1038/nmat4009
232. Tung JC, Barnes JM, Desai SR, Sistrunk C, Conklin MW, Schedin P, et al. Tumor mechanics and metabolic dysfunction. *Free Radic Biol Med* (2015) 79:269–80. doi: 10.1016/j.freeradbiomed.2014.11.020
233. Masuzaki R, Tateishi R, Yoshida H, Sato T, Ohki T, Goto T, et al. Assessing liver tumor stiffness by transient elastography. *Hepatol Int* (2007) 1(3):394–7. doi: 10.1007/s12072-007-9012-7
234. Lopez JI, Kang I, You WK, McDonald DM, Weaver VM. In situ force mapping of mammary gland transformation. *Integr Biol (Camb)* (2011) 3 (9):910–21. doi: 10.1039/c1ib00043h
235. Pang M, Teng Y, Huang J, Yuan Y, Lin F, Xiong C. Substrate stiffness promotes latent TGF-beta1 activation in hepatocellular carcinoma. *Biochem Biophys Res Commun* (2017) 483(1):553–8. doi: 10.1016/j.bbrc.2016.12.107
236. Pang MF, Siedlik MJ, Han S, Stallings-Mann M, Radisky DC, Nelson CM. Tissue Stiffness and Hypoxia Modulate the Integrin-Linked Kinase ILK to Control Breast Cancer Stem-like Cells. *Cancer Res* (2016) 76(18):5277–87. doi: 10.1158/0008-5472.CAN-16-0579
237. Egeblad M, Rasch MG, Weaver VM. Dynamic interplay between the collagen scaffold and tumor evolution. *Curr Opin Cell Biol* (2010) 22(5):697–706. doi: 10.1016/j.ceb.2010.08.015
238. Lu P, Weaver VM, Werb Z. The extracellular matrix: a dynamic niche in cancer progression. *J Cell Biol* (2012) 196(4):395–406. doi: 10.1083/jcb.201102147
239. Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell* (2009) 139(5):891–906. doi: 10.1016/j.cell.2009.10.027
240. Pickup MW, Laklai H, Acerbi I, Owens P, Gorska AE, Chytil A, et al. Stromally derived lysyl oxidase promotes metastasis of transforming growth factor-beta-deficient mouse mammary carcinomas. *Cancer Res* (2013) 73 (17):5336–46. doi: 10.1158/0008-5472.CAN-13-0012
241. Rubashkin MG, Cassereau L, Bainer R, DuFort CC, Yui Y, Ou G, et al. Force engages vinculin and promotes tumor progression by enhancing PI3K activation of phosphatidylinositol (3,4,5)-triphosphate. *Cancer Res* (2014) 74(17):4597–611. doi: 10.1158/0008-5472.CAN-13-3698
242. Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, et al. Tensional homeostasis and the malignant phenotype. *Cancer Cell* (2005) 8 (3):241–54. doi: 10.1016/j.ccr.2005.08.010
243. Zhao B, Wei X, Li W, Udan RS, Yang Q, Kim J, et al. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev* (2007) 21(21):2747–61. doi: 10.1101/gad.1602907
244. Pavel M, Renna M, Park SJ, Menzies FM, Ricketts T, Fullgrabe J, et al. Contact inhibition controls cell survival and proliferation via YAP/TAZ-autophagy axis. *Nat Commun* (2018) 9(1):2961. doi: 10.1038/s41467-018-05388-x
245. Wang Z, Li C, Jiang M, Chen J, Yang M, Pu J, et al. (FLNA) regulates autophagy of bladder carcinoma cell and affects its proliferation, invasion and metastasis. *Int Urol Nephrol* (2018) 50(2):263–73. doi: 10.1007/s11255-017-1772-y
246. Sun GG, Lu YF, Zhang J, Hu WN. Filamin A regulates MMP-9 expression and suppresses prostate cancer cell migration and invasion. *Tumour Biol* (2014) 35(4):3819–26. doi: 10.1007/s13277-013-1504-6
247. Krebs K, Ruusmann A, Simonlatser G, Velling T. Expression of FLNa in human melanoma cells regulates the function of integrin alpha1beta1 and phosphorylation and localisation of PKB/AKT/ERK1/2 kinases. *Eur J Cell Biol* (2015) 94(12):564–75. doi: 10.1016/j.ejcb.2015.10.006
248. Ulbricht A, Arndt V, Hohfeld J. Chaperone-assisted proteostasis is essential for mechanotransduction in mammalian cells. *Commun Integr Biol* (2013) 6 (4):e24925. doi: 10.4161/cib.24925

249. Sanhueza C, Wehinger S, Castillo Bennett J, Valenzuela M, Owen GI, Quest AF. The twisted survivin connection to angiogenesis. *Mol Cancer* (2015) 14:198. doi: 10.1186/s12943-015-0467-1
250. Yang H, Guan L, Li S, Jiang Y, Xiong N, Li L, et al. Correction: Mechanosensitive caveolin-1 activation-induced PI3K/Akt/mTOR signaling pathway promotes breast cancer motility, invadopodia formation and metastasis in vivo. *Oncotarget* (2018) 9(66):32730. doi: 10.18632/oncotarget.26065
251. Nah J, Yoo SM, Jung S, Jeong EI, Park M, Kaang BK, et al. Phosphorylated CAV1 activates autophagy through an interaction with BECN1 under oxidative stress. *Cell Death Dis* (2017) 8(5):e2822. doi: 10.1038/cddis.2017.71
252. Quest AF, Gutierrez-Pajares JL, Torres VA. Caveolin-1: an ambiguous partner in cell signalling and cancer. *J Cell Mol Med* (2008) 12(4):1130–50. doi: 10.1111/j.1582-4934.2008.00331.x
253. Bender FC, Reymond MA, Bron C, Quest AF. Caveolin-1 levels are down-regulated in human colon tumors, and ectopic expression of caveolin-1 in colon carcinoma cell lines reduces cell tumorigenicity. *Cancer Res* (2000) 60 (20):5870–8.
254. Simon L, Campos A, Leyton L, Quest AFG. Caveolin-1 function at the plasma membrane and in intracellular compartments in cancer. *Cancer Metastasis Rev* (2020) 39(2):435–53. doi: 10.1007/s10555-020-09890-x
255. Diaz-Valdivia N, Bravo D, Huerta H, Henriquez S, Gabler F, Vega M, et al. Enhanced caveolin-1 expression increases migration, anchorage-independent growth and invasion of endometrial adenocarcinoma cells. *BMC Cancer* (2015) 15:463. doi: 10.1186/s12885-015-1477-5
256. Fabbri L, Bost F, Mazure NM. Primary Cilium in Cancer Hallmarks. *Int J Mol Sci* (2019) 20(6):1336. doi: 10.3390/ijms20061336
257. Ko JY, Lee EJ, Park JH. Interplay Between Primary Cilia and Autophagy and Its Controversial Roles in Cancer. *Biomol Ther (Seoul)* (2019) 27(4):337–41. doi: 10.4062/biomolther.2019.056
258. Liu L, Sheng JQ, Wang MR, Gan Y, Wu XL, Liao JZ, et al. Primary Cilia Blockage Promotes the Malignant Behaviors of Hepatocellular Carcinoma via Induction of Autophagy. *BioMed Res Int* (2019) 2019:5202750. doi: 10.1155/2019/5202750
259. Shimizu S, Kanaseki T, Mizushima N, Mizuta T, Arakawa-Kobayashi S, Thompson CB, et al. Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. *Nat Cell Biol* (2004) 6(12):1221–8. doi: 10.1038/ncb1192
260. Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, et al. Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science* (2004) 304(5676):1500–2. doi: 10.1126/science.1096645
261. Espert L, Denizot M, Grimaldi M, Robert-Hebmann V, Gay B, Varbanov M, et al. Autophagy is involved in T cell death after binding of HIV-1 envelope proteins to CXCR4. *J Clin Invest* (2006) 116(8):2161–72. doi: 10.1172/JCI26185
262. Mukhopadhyay S, Panda PK, Sinha N, Das DN, Bhutia SK. Autophagy and apoptosis: where do they meet? *Apoptosis* (2014) 19(4):555–66. doi: 10.1007/s10495-014-0967-2
263. Wang K, Wei Y, Liu W, Liu L, Guo Z, Fan C, et al. Mechanical Stress-Dependent Autophagy Component Release via Extracellular Nanovesicles in Tumor Cells. *ACS Nano* (2019) 13(4):4589–602. doi: 10.1021/acsnano.9b00587
264. Gonzalez CD, Resnik R, Vaccaro MI. Secretory Autophagy and Its Relevance in Metabolic and Degenerative Disease. *Front Endocrinol (Lausanne)* (2020) 11:266. doi: 10.3389/fendo.2020.00266
265. Zhang L, Zhang S, Yao J, Lowery FJ, Zhang Q, Huang WC, et al. Microenvironment-induced PTEN loss by exosomal microRNA primes brain metastasis outgrowth. *Nature* (2015) 527(7576):100–4. doi: 10.1038/nature15376
266. Zomer A, Maynard C, Verweij FJ, Kamermans A, Schafer R, Beerling E, et al. In Vivo imaging reveals extracellular vesicle-mediated phenocopying of metastatic behavior. *Cell* (2015) 161(5):1046–57. doi: 10.1016/j.cell.2015.04.042
267. Sandilands E, Serrels B, McEwan DG, Morton JP, Macagno JP, McLeod K, et al. Autophagic targeting of Src promotes cancer cell survival following reduced FAK signalling. *Nat Cell Biol* (2011) 14(1):51–60. doi: 10.1038/ncb2386
268. Arjonen A, Alanko J, Veltel S, Ivaska J. Distinct recycling of active and inactive beta1 integrins. *Traffic* (2012) 13(4):610–25. doi: 10.1111/j.1600-0854.2012.01327.x
269. Rainero E, Howe JD, Caswell PT, Jamieson NB, Anderson K, Critchley DR, et al. Ligand-Occupied Integrin Internalization Links Nutrient Signaling to Invasive Migration. *Cell Rep* (2015) 10(3):398–413. doi: 10.1016/j.celrep.2014.12.037
270. Colombero C, Remy D, Antoine S, Macé A-S, Monteiro P, ElKhatib N, et al. mTOR repression in response to amino acid starvation promotes ECM degradation through MT1-MMP endocytosis arrest. *bioRxiv* (2021). doi: 10.1101/2021.01.29.428784
271. Yoon SO, Shin S, Mercurio AM. Hypoxia stimulates carcinoma invasion by stabilizing microtubules and promoting the Rab11 trafficking of the alpha6beta4 integrin. *Cancer Res* (2005) 65(7):2761–9. doi: 10.1158/0008-5472.CAN-04-4122
272. Nguyen CDK, Yi C. YAP/TAZ Signaling and Resistance to Cancer Therapy. *Trends Cancer* (2019) 5(5):283–96. doi: 10.1016/j.trecan.2019.02.010
273. Thompson BJ. YAP/TAZ: Drivers of Tumor Growth, Metastasis, and Resistance to Therapy. *Bioessays* (2020) 42(5):e1900162. doi: 10.1002/bies.201900162
274. Jutten B, Keulers TG, Schaaf MB, Savelkouls K, Theys J, Span PN, et al. EGFR overexpressing cells and tumors are dependent on autophagy for growth and survival. *Radiother Oncol* (2013) 108(3):479–83. doi: 10.1016/j.radonc.2013.06.033
275. Machado-Neto JA, Coelho-Silva JL, Santos FPS, Scheucher PS, Campregher PV, Hamerschlak N, et al. Autophagy inhibition potentiates ruxolitinib-induced apoptosis in JAK2(V617F) cells. *Invest New Drugs* (2020) 38(3):733–45. doi: 10.1007/s10637-019-00812-5
276. Chauhan VP, Martin JD, Liu H, Lacorre DA, Jain SR, Kozin SV, et al. Angiotensin inhibition enhances drug delivery and potentiates chemotherapy by decompressing tumour blood vessels. *Nat Commun* (2013) 4:2516. doi: 10.1038/ncomms3516
277. Woo Y, Jung YJ. Angiotensin II receptor blockers induce autophagy in prostate cancer cells. *Oncol Lett* (2017) 13(5):3579–85. doi: 10.3892/ol.2017.5872
278. Hernandez-Caceres MP, Cereceda K, Hernandez S, Li Y, Narro C, Rivera P, et al. Palmitic acid reduces the autophagic flux in hypothalamic neurons by impairing autophagosome-lysosome fusion and endolysosomal dynamics. *Mol Cell Oncol* (2020) 7(5):1789418. doi: 10.1080/23723556.2020.1789418
279. Hernandez-Caceres MP, Toledo-Valenzuela L, Diaz-Castro F, Avalos Y, Burgos P, Narro C, et al. Palmitic Acid Reduces the Autophagic Flux and Insulin Sensitivity Through the Activation of the Free Fatty Acid Receptor 1 (FFAR1) in the Hypothalamic Neuronal Cell Line N43/5. *Front Endocrinol (Lausanne)* (2019) 10:176. doi: 10.3389/fendo.2019.00176
280. Grenci G, Bertocchi C, Ravasio A. Integrating Microfabrication into Biological Investigations: the Benefits of Interdisciplinarity. *Micromachines (Basel)* (2019) 10(4):252. doi: 10.3390/mi10040252
281. Bertocchi C, Goh WI, Zhang Z, Kanchanawong P. Nanoscale imaging by superresolution fluorescence microscopy and its emerging applications in biomedical research. *Crit Rev Biomed Eng* (2013) 41(4-5):281–308. doi: 10.1615/critrevbiomedeng.2014010448

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Hernández-Cáceres, Munoz, Pradenas, Pena, Lagos, Aceiton, Owen, Morselli, Criollo, Ravasio and Bertocchi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Oncology

MINI REVIEW
published: 26 February 2021
doi: 10.3389/fonc.2021.616079

Mitophagy in Pancreatic Cancer

Yangchun Xie[1]*, *Jiao Liu*[2], *Rui Kang*[3] and *Daolin Tang*[3]*

[1] Department of Oncology, The Second Xiangya Hospital, Central South University, Changsha, China, [2] The Third Affiliated Hospital, Guangzhou Medical University, Guangzhou, China, [3] Department of Surgery, UT Southwestern Medical Center, Dallas, TX, United States

Pancreatic ductal adenocarcinoma (PDAC), one of the most aggressive solid malignancies, is characterized by the presence of oncogenic KRAS mutations, poor response to current therapies, prone to metastasis, and a low 5-year overall survival rate. Macroautophagy (herein referred to as autophagy) is a lysosome-dependent degradation system that forms a series of dynamic membrane structures to engulf, degrade, and recycle various cargoes, such as unused proteins, damaged organelles, and invading pathogens. Autophagy is usually upregulated in established cancers, but it plays a dual role in the regulation of the initiation and progression of PDAC. As a type of selective autophagy, mitophagy is a mitochondrial quality control mechanism that uses ubiquitin-dependent (e.g., the PINK1-PRKN pathway) and -independent (e.g., BNIP3L/NIX, FUNDC1, and BNIP3) pathways to regulate mitochondrial turnover and participate in the modulation of metabolism and cell death. Genetically engineered mouse models indicate that the loss of PINK1 or PRKN promotes, whereas the depletion of BNIP3L inhibits oncogenic KRAS-driven pancreatic tumorigenesis. Mitophagy also play a dual role in the regulation of the anticancer activity of certain cytotoxic agents (e.g., rocaglamide A, dichloroacetate, fisetin, and P. suffruticosa extracts) in PDAC cells or xenograft models. In this min-review, we summarize the latest advances in understanding the complex role of mitophagy in the occurrence and treatment of PDAC.

Keywords: mitophagy, autophagy, PDAC - pancreatic ductal adenocarcinoma, tumorigenesis, therapy

OPEN ACCESS

Edited by:
Eugenia Morselli, Pontificia Universidad Católica de Chile, Chile

Reviewed by:
Maria Markaki, Foundation for Research and Technology Hellas (FORTH), Greece
Patricia V. Burgos, San Sebastián University, Chile

***Correspondence:**
Yangchun Xie
xieyangchun88@csu.edu.cn
Daolin Tang
daolin.tang@utsouthwestern.edu

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *11 October 2020*
Accepted: *22 January 2021*
Published: *26 February 2021*

Citation:
Xie Y, Liu J, Kang R and Tang D (2021) Mitophagy in Pancreatic Cancer. Front. Oncol. 11:616079. doi: 10.3389/fonc.2021.616079

INTRODUCTION

More than 90% of pancreatic cancers are ductal adenocarcinoma (PDAC), which is highly malignant, difficult to diagnose early, and has a very poor prognosis. It is estimated that by 2030, pancreatic cancer will become the second largest tumor-related death in humans (1). Although there have been a variety of "precision" targeted therapies for certain solid cancers (such as lung and breast cancer), the clinical treatment of PDAC is still in the "non-precision" era. The effective rate of the widely used gemcitabine regimen is only 30%, while FOLFIRINOX (a regimen consisting of 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin) has serious adverse reactions, and the targeted drug erlotinib (an oral tyrosine kinase inhibitor of epidermal growth factor receptor [EGFR]) as well as cutting-edge immune checkpoint inhibitors have limited efficacy in patients with PDAC (2). How to achieve "precise" diagnosis and treatment of PDAC is a challenging issue in clinical practice. This clinical goal may require in-depth basic research to understand the complex pathological mechanisms of PDAC initiation and development.

Cells produce a large amount of waste every day, which needs to be removed by an integrated degradation system to maintain normal cell functions. In addition to the ubiquitin-proteasome system (UPS), autophagy is a lysosomal-dependent pathway that can remove various endogenous cellular materials (such as proteins and organelles) and exogenous invading pathogens. Autophagy dysfunction (including defects or over-activation) may cause abnormal cell components and functions, leading to various pathological conditions and diseases (3). Therefore, it is important to understand the types, functions, and regulation of autophagy under different conditions (4). The focus on autophagy provides a promising alternative to the development of new treatment options for human diseases. In this min-review, we describe the types of autophagy and the mechanism of mitophagy, and then analyze the effects of mitophagy on PDAC, including tumorigenesis and tumor treatment.

TYPE OF AUTOPHAGY

According to the different ways of transporting cellular components to lysosomes, autophagy is divided into the following categories (**Figure 1A**) (4). 1) Macroautophagy. The process of macroautophagy is a dynamic membrane reforming process involving the formation and maturation of three special structures: phagophore (also known as separated membrane produced by endoplasmic reticulum, mitochondria, or other subcellular membrane organelles), autophagosome (a double-membrane organelle phagocytosing degradable materials), and autolysosome (a hybrid organelle formed by the fusion of autophagosomes and lysosomes) where sequestered material is degraded by lysosomal hydrolases. 2) Microautophagy: lysosome membrane directly envelops longevity protein and then degrades in lysosome; 3) Chaperone-mediated autophagy (5): proteins containing KFERQ-like motifs bind to molecular chaperones (such as heat shock protein family A (Hsp70) member 8 [HSPA8/HSC70]), and then are transported to the lysosome cavity by lysosomal associated membrane protein 2 (LAMP2/LAMP2A) to be digested by lysosomal enzymes. It is worth noting that the multimerization of LAMP2 is required to transport the substrate into the lysosomal cavity (6, 7). Among them, macroautophagy (hereinafter referred to as autophagy) is the most common and well-studied form of autophagy in mammalian cells. The so-called autophagy-related (ATG) genes or proteins play a key role in the regulation of autophagy membrane dynamics through protein-protein interaction, and post-translational modifications (especially phosphorylation) further regulate autophagic process by affecting ATG function (8).

According to the selectivity of the substrate to be degraded, autophagy is further divided into selective autophagy and non-selective autophagy to control cell fate (9, 10) (**Figure 1A**). Non-selective autophagy refers to non-specific degradation processes, such as starvation-induced autophagic degradation. In addition to the core autophagy mechanism, selective autophagy also requires specific autophagy receptors to selectively degrade specific cargo (9, 11, 12). For example, xenophagy (13), clockophagy (14, 15), and mitophagy (16) can selectively degrade invading pathogens, aggregated circadian protein aryl hydrocarbon receptor nuclear translocator like (ARNTL), and damaged mitochondria, respectively (**Figure 1A**). This selective autophagy mainly depends on the molecular bridge-like autophagy receptor (also called adaptor protein), which not only specifically binds to the substrate, but also binds to members of the ATG8/LC3 family (MAP1LC3A, MAP1LC3B, MAP1LC3C, GABARAP, GABARAPL1/GEC1, GABARAPL2/GATE-16, and GABARAPL3) through different structure domains (12). The number of genes in the ATG8/LC3 family may be caused by gene duplication and loss events during evolution. LC3-II is a standard marker for autophagosomes, which is produced by conjugating cytoplasmic LC3-I with phosphatidylethanolamine on the surface of newborn autophagosomes (17). It is worth noting that certain autophagy receptors (such as sequestosome 1 [SQSTM1/p62]) act on both selective and non-selective autophagy during stress (18). In addition, the protein level of SQSTM1 is also regulated by the crosstalk between the UPS and autophagy pathways (19). Collectively, these kinetics indicate that a complex feedback network is involved in metabolism and signal transduction to control substrate degradation (20).

TYPE OF MITOPHAGY

Mitochondria are organelles composed of two membranes (inner membrane and outer membrane) found in most cells. Normal mitochondria act as a "power factory" whose main function is to perform aerobic respiration to produce adenosine triphosphate (ATP). In addition, the interaction between mitochondrial and non-mitochondrial metabolic pathways is important for generating secondary signals (such as reactive oxygen species [ROS] and calcium) and biological macromolecules (such as proteins, carbohydrates, lipids, and nucleic acids). Therefore, maintaining healthy mitochondria, including quantity and quality, is essential for cell homeostasis. Conversely, damage to the mitophagy pathway can cause various pathological conditions (such as inflammation) and diseases (such as neurodegenerative diseases and cancer) (21–23). As an important component of the mitochondrial quality control mechanism, mitophagy can be activated through either ubiquitin (Ub)-dependent or independent pathway (**Figure 1A**), which is regulated by various proteins, including mitochondrial inner or outer membrane proteins (**Figure 1B**).

Mitophagy that rely on Ub can be further divided into classical and non-classical pathways (24). The classical pathway is mediated by the PTEN induced kinase 1 (PINK1, a serine–threonine protein kinase) and parkin RBR E3 ubiquitin protein ligase (PRKN/PARK2) (25). Mutations in PINK1 and PRKN are one of the important causes of Parkinson's disease, a progressive neurodegenerative disease with motor and non-motor symptoms. The impaired PINK1-PRKN-dependent mitophagy pathway also promotes various types of tumor formation, including PDAC (discussed later). Mechanistically, oxidative damage to the mitochondria causes the accumulation of PINK1 on the mitochondrial outer membrane and the recruitment of PRKN

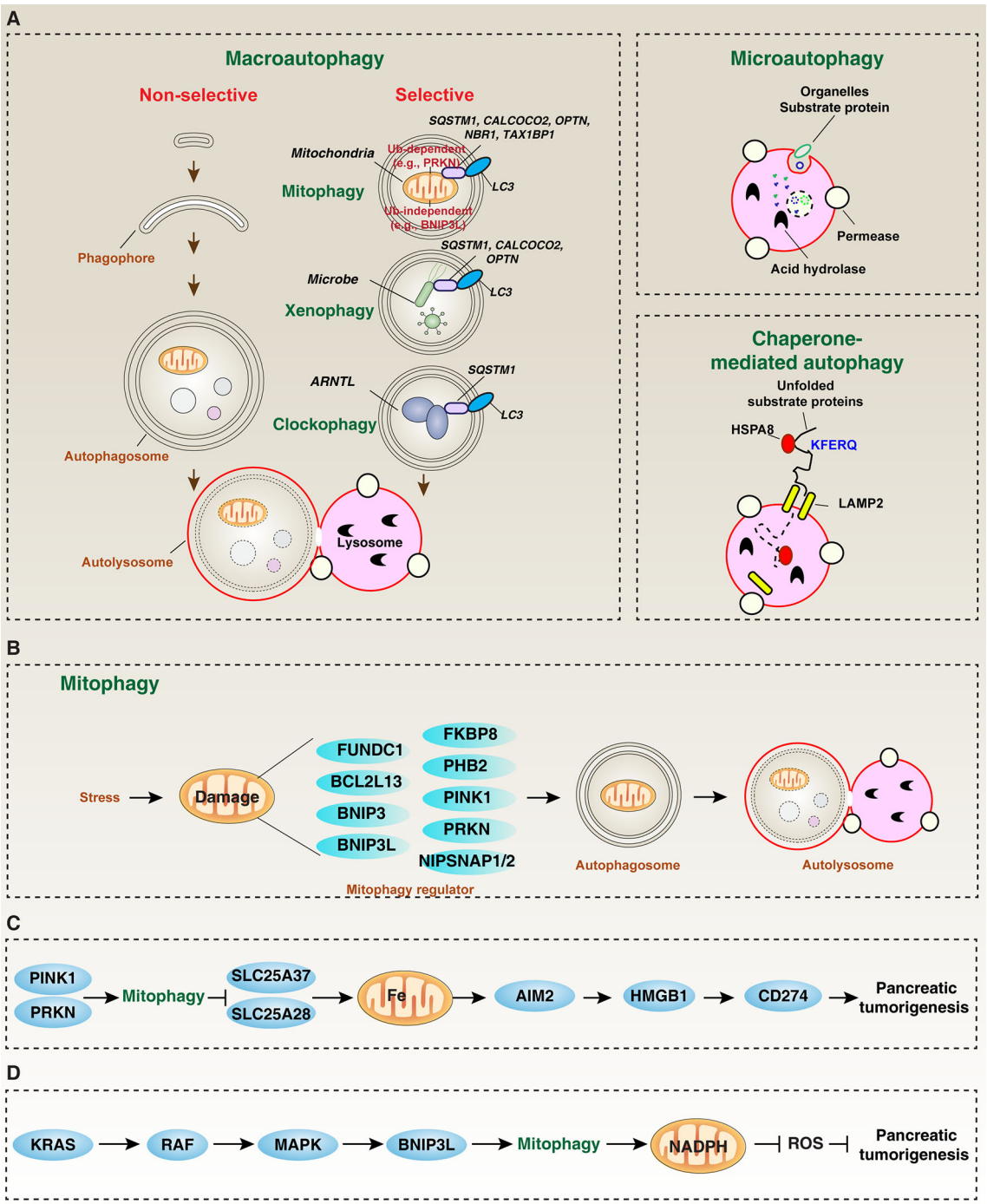


FIGURE 1 | The role of mitophagy in pancreatic tumorigenesis. **(A)** In mammalian cells, there are three main types of autophagy: microautophagy, macroautophagy, and chaperone-mediated autophagy. Macroautophagy can be further divided into selective and non-selective forms. **(B)** Core mitophagy regulators mediate mitochondrial clearance. **(C, D)** PINK1/PRKN and BNIP3L-dependent mitophagy play different roles in inhibiting or promoting pancreatic tumorigenesis, respectively.

from the cytoplasm to the mitochondria, leading to subsequent assembly of phosphorylated Ub chains on mitochondrial outer membrane proteins (25). In addition to the earliest reported SQSTM1 (25), other autophagy receptors, such as optineurin (OPTN) (26), neighbor of BRCA1 gene 1 (NBR1) (27), calcium binding and coiled-coil domain 2 (CALCOCO2/NDP52) (28), and tax1 binding protein 1 (TAX1BP1) (28), also help to recognize and degrade damaged mitochondria after activating

the PINK1-PRKN pathway (**Figure 1A**). Moreover, PINK1-PRKN-mediated mitophagy can be reversed by deubiquitinating enzymes, such as ubiquitin-specific peptidase 8 (USP8), USP15, USP30, and USP35 (29). Non-classical Ub-dependent mitophagy is mediated by non-PRKN E3 ubiquitin ligases (such as mitochondrial E3 ubiquitin protein ligase 1 [MUL1] (30), siah E3 ubiquitin protein ligase 1 [SIAH1] (31), SMAD specific E3 ubiquitin protein ligase 1 [SMURF1] (32), and autocrine motility factor receptor [AMFR/GP78]) (33). The impact of crosstalk between Ub-dependent classical and non-classical mitophagy pathways on tumors is still poorly understood.

Ub-independent mitophagy is mediated by receptors, rather than E3 ligases. Recently, depending on the stimulus and cell type, the list of mitophagy receptors is increasing (34). In addition to the early reports of BCL2 interacting protein 3 like (BNIP3L/NIX) acting as a mitophagy receptor in red cells (35), other mitophagy receptors, including FUN14 domain containing 1 (FUNDC1) (36), BCL2 interacting protein 3 (BNIP3) (37), nipsnap homolog 1 (NIPSNAP1) (38), nipsnap homolog 2 (NIPSNAP2) (38), prohibitin 2 (PHB2) (39), BCL2 like 13 (BCL2L13) (40) and FKBP prolyl isomerase 8 (FKBP8) (41), have also been identified in cancer and non-cancer cells (**Figure 1B**). These unique receptors are responsible for binding to different mitochondrial membrane components in response to various stresses (such as hypoxia and oxidative damage). In addition to protein, non-protein mitochondrial components, such as cardiolipin and ceramide (42), also mediate mitophagy in some case, indicating that there are complex mitophagy sub-routes to regulate mitochondrial turnover and function.

MITOPHAGY IN PANCREATIC CANCER

Compared to normal cells, pancreatic cancer cells generally exhibit highly fragmented mitochondria, which is associated with increased mitochondrial fission and numbers as well as enhanced mitochondrial oxidative phosphorylation or glycolysis (43–45). Therefore, understanding the mechanism of mitochondrial biogenesis and turnover in different stages of pancreatic cancer, including initiation, progression, and metastasis, is essential for the next generation of cancer treatments. Indeed, increased autophagy or mitophagy levels are observed in various types of pancreatic cancer (46–48). However, autophagy plays a dual role in various cancer (including PDAC), depending on many factors, such as tumor stage, tumor microenvironment, gene mutation status involving oncogenes and tumor suppressor genes, and metabolic reprogramming (49–54). PDAC is a heterogeneous disease and can be morphologically classified into four types: conventional, tubulo-papillary, squamous, and "composite", which exhibit different molecular and genetic characteristics (55). Generally, autophagy inhibits the growth of PDAC in the early stage by limiting DNA damage or inflammation, and upregulated autophagy in the later stage can promote PDAC survival by limiting cell death or anti-tumor immunity (56–60). Since covering all the effects of autophagy in PDAC is outside the scope of this min-review, we will only discuss the modulation and function of mitophagy in PDAC as described below.

Pancreatic Tumorigenesis

Evidence is accumulating that both intrinsic genetic factor and extrinsic environmental factor are important for tumorigenesis. For pancreatic cancer, the oncogenic KRAS mutation is a key driving force for the formation of precursor lesions and subsequent development of PDAC with stromal response (61). KRAS activation is related to changes in mitochondrial morphology (e.g., increased mitochondrial fragmentation) and function (for example, reduction of mitochondrial respiratory complex I activity, enhancement of glycolytic activity, promotion of ROS production and induction of mitophagy) in various cancers (including PDAC) (62–66). Moreover, the conditional expression of endogenous $Kras^{G12D}$ in the pancreas of mice (*Pdx1-Cre;*$Kras^{G12D}$; called KC mice) can mimic most of pathological development of human PDAC (67). This spontaneous transgenic PDAC mouse model is widely used to further consume or overexpress additional genes to evaluate the function of target genes in pancreatic tumorigenesis (**Table 1**). For example, based on KC mice, further depletion of the tumor suppressor high mobility group box 1 (HMGB1, *Pdx1-Cre;*$Kras^{G12D}$*;*$Hmgb1^{-/-}$) (69) or overexpression of tumor protein p53 (TP53) mutation (*Pdx1-Cre;*$Kras^{G12D}$*;*$Tp53^{R172H}$, termed KPC mice) (70) can significantly promote the development of KRAS-driven PDAC. HMGB1 is a positive regulator of autophagy and mitophagy, coupled with TP53 signaling in a variety of tumors (71–74). Cytoplasmic HMGB1 is a BECN1-binding protein that contributes to the formation of autophagosomes (72). Nuclear HMGB1 promotes the expression of heat shock protein β-1 (HSPB1) and subsequent HSPB1-mediated cytoskeletal integrity, which is required for the membrane dynamics of mitophagy (71). In addition, mitochondrial HMGB1 repairs mitochondrial genomic DNA damage, which also plays a potential role in suppressing tumorigenesis (75). However, depletion of mitophagy regulators, including PINK1 [*Pdx1-Cre;*$Kras^{G12D}$*;*$Pink1^{-/-}$] (22), PRKN [*Pdx1-Cre;*$Kras^{G12D}$*;*$Prkn^{-/-}$] (22), or BNIP3L/NIX [*Pdx1-Cre;*$Kras^{G12D}$*;*$Bnip3l^{-/-}$ *or*

TABLE 1 | Mitophagy regulators in PDAC.

Mitophagy regulator	Expression in human PDAC	Function	Mechanism	Refs
BNIP3L	Upregulation	Tumor promoter	Increases glucose metabolism and antioxidant capacity	(68)
PINK1	Upregulation	Tumor suppressor	Inhibits inflammation and mitochondrial iron-related antitumor immunity	(22)
PRKN	Downregulation	Tumor suppressor	Inhibits inflammation and mitochondrial iron-related antitumor immunity	(22)
HMGB1	Upregulation	Tumor suppressor	Inhibits genomic instability and mitochondrial dysfunction	(69)
TP53	Upregulation	Tumor suppressor	Inhibits genomic instability and mitochondrial dysfunction	(70)

Pdx1-Cre;Kras^{G12D}*;Tp53*^{R172H}*; Bnip3l*$^{-/-}$ (68), in KC mice exhibits different phenotype in pancreatic tumorigenesis. These transgenic animal studies show that Ub-dependent and independent mitophagy pathways play different roles in PDAC.

Dysregulated autophagy promotes or inhibits the growth of pancreatic cancer by interfering with different metabolic pathways or tumor signals, such as carbohydrate metabolism, fatty acid β-oxidation, and amino acid transport (48). For example, the reduced glycolysis gene PKM2 promotes survival by maintaining autophagy induced by low glucose in PDAC cells (76). Autophagy-mediated lipid degradation and subsequent fatty acid β-oxidation may provide additional resources for ATP production during PDAC growth (77, 78). Autophagy-mediated degradation of cellular material provides reusable amino acids for PDAC cell proliferation during glutamine deprivation (79). In addition, the PINK1-PRKN pathway can degrade mitochondrial iron importers (such as solute carrier family 25 member 37 [SLC25A37] and solute carrier family 25 member 28 [SLC25A28]) through SQSTM1-mediated mitophagy to inhibit carcinogenic KRAS-driven pancreatic tumorigenesis in mice, thereby inhibiting mitochondrial iron-mediated absent in melanoma 2 (AIM2)-dependent inflammasome activation and the subsequent activation of damage associated molecular pattern (DAMP, such as HMGB1)-dependent immune checkpoint expression (e.g., CD274/PD-L1) (**Figure 1C**) (22). These findings establish a role of PINK1/PRKN-mediated mitophagy to inhibit pancreatic tumorigenesis by limiting chronic inflammation-related immunosuppression in the hypoxic tumor microenvironment (80). Of note, high expression of *PRKN* mRNA was found to be associated with improved survival of pancreatic cancer patients, whereas mRNA expression of *PINK1* did not influence patient survival (22), indicating that PINK1 is a contributor of PDAC, but it is not a potential biomarker. In addition, both PINK1 and PRKN may have mitophagy-independent functions in controlling the quality of mitochondria during pancreatic tumorigenesis (22).

In contrast, in a precursor lesion called pancreatic intraepithelial neoplasia (PanIN), oncogenic KRAS-mediated BNIP3L expression may activate mitophagy in a rapidly accelerated fibrosarcoma (RAF)-mitogen-activated protein kinase (MAPK)-dependent manner to limit the flux of glucose to mitochondria and enhance reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent redox capacity, thereby promoting pancreatic tumorigenesis (**Figure 1D**) (68). In KC and KPC pancreatic cancer models, the depletion of additional BNIP3L will increase the content of mitochondria in PanIN, thereby increasing the production of mitochondrial ROS to limit the development of PanIN to PDAC (68). These observations indicate that BNIP3L-mediated mitophagy have different roles in promoting pancreatic tumorigenesis by enhancing the antioxidant capacity of cancer cells for cell proliferation and metastasis. However, oxidative stress and redox regulation are double-edged swords in tumorigenesis (81). Certain types of oxidative cell death, such as necroptosis (a caspase-independent regulated necrosis) and ferroptosis (an iron-dependent regulated necrosis), can promote KRAS-driven PDAC by activating inflammation-related immune suppression (82–84). Whether PINK1, PRKN2, and BNIP3L have non-mitochondrial functions in the modulation of the oncogene KRAS signal remains to be seen. In addition, various types of regulated cell death are closely related to autophagy (85–87), which may accelerate the complexity of the immune characteristics of the tumor microenvironment, thereby affecting anti-tumor immunity.

There is emerging evidence that impaired mitophagy is related to epithelial-mesenchymal transition and pancreatic cancer stem cells (pCSCs), which are pluripotent, self-renewable, and capable of forming tumors (88). In particular, the interferon signaling pathway-mediated the upregulation of Ub-like modifier interferon-stimulated gene 15 (ISG15) and its modification ISGylation maintain mitophagy and metabolic plasticity of pCSCs (89), suggesting a potential link between interferon, mitophagy, and metabolism in pCSCs. PDAC patients with high ISG15 levels showed increased expression of genes related to the CSC pathway, including epithelial-mesenchymal transition and oxidative phosphorylation (89). In contrast, the inhibition of ISG15/ISGylation impairs PRKN-dependent mitophagy, causing pCSCs to fail to eliminate dysfunctional and unhealthy mitochondria (89). Overall, these findings support a role of ISG15 in pCSCs by regulating mitochondrial dynamics and energy metabolism. The role of ISG15 in pancreatic tumorigenesis needs to be further studied using transgenic mice.

Pancreatic Cancer Therapy

The purpose of tumor treatment is to induce death in tumor cells without damaging normal cells. Cell death can be divided into accidental or regulated cell death (90). Regulated cell death further includes apoptotic and non-apoptotic forms. In addition to the extensively studied apoptosis (91, 92), the induction of non-apoptotic regulated cell death [such as necroptosis (93, 94), alkaliptosis (95, 96), and ferroptosis (97–101)] in preclinical PDAC models has shown promising results in inhibiting tumor growth. As a metabolic center, mitochondria play a complex role in regulating apoptosis and non-apoptotic cell death by cooperating with other subcellular organelles (102). Accordingly, mitophagy-mediated mitochondrial degradation and turnover is reasonable to affect the anti-cancer activity of cytotoxic agents in PDAC cells. The best cell models for studying mitochondrial biology and mitophagy of pancreatic cancer are various human PDAC cell lines with KRAS mutations. For example, rocaglamide A, a natural product from the plant Aglaia elliptifolia, has the ability to induce PINK1/PRKN-mediated mitophagy as a negative feedback mechanism to limit rocaglamide A-induced apoptosis in various human PDAC cell lines with KRAS mutations (103). In contrast, the inhibition of mitophagy by Mdivi-1 enhances the anti-cancer activity of rocaglamide A in PDAC cells (103). Overexpression of serine/threonine kinase 25 (STK25, also known as MST1) in various PDAC cells induces apoptosis by inhibiting mitophagy mediated by mitofusin 2 (MFN2) (104). In contrast, leflunomide, an FDA-approved arthritis drug, can inhibit the growth of PDAC tumors by inducing MFN2 expression and subsequent mitophagy (44). In addition, *in vitro* and xenograft models, the combination of cyst(e)inase (an engineered human enzyme) and anuranofin (a thioredoxin reductase inhibitor) can inhibit mitophagy, thereby increase ROS production and apoptosis in the human PDAC cells (105). In other cases, dichloroacetate (an inhibitor of pyruvate

dehydrogenase kinase) (106), fisetin (a bioactive flavonoid molecule found in fruits and vegetables) (107), and P. suffruticosa extracts (108) may play a context-related role in the induction of mitophagy and tumor suppression in PDAC cells. These findings further indicate that the complex relationship between mitophagy and mitochondrial dynamics can affect the effects of chemotherapy and targeted therapy.

In addition to PDAC cells, pCSCs is another cell model for studying mitochondrial dysfunction. pCSCs not only promote the growth and metastasis of pancreatic tumors, but also mediate chemoresistance. Metformin is a biguanide anti-diabetic drug that activates AMP-activated protein kinase (AMPK) to trigger autophagy (109). Retrospective studies have shown that compared with patients receiving insulin or sulfonylureas, many diabetic patients with solid tumors (including pancreatic cancer) treated with metformin have a survival benefit (109). The loss of ISG15 in pCSCs by CRISPR-Cas9 technology results in sensitivity to metformin therapy in xenograft models (89). These findings further indicate a potential role of ISG15 in regulating the anti-cancer activity of metformin in pCSCs. Further investigations are still needed to determine whether ISG15 directly regulates AMPK activation in pCSCs.

CONCLUSION AND PERSPECTIVES

In the past decade, basic and clinical research on autophagy has involved various diseases, including pancreatic cancer (48, 59, 110–112). With the deepening of research, the functions of autophagy in tumor biology show diversity and complexity. One of the important reasons is that autophagy can have different degradation substrates, and these substrates can play a tumor-promoting and anti-tumor effect. In addition, the degree of substrate degradation (such as complete or partial degradation) also affects the function of autophagy in tumors. Similarly, mitochondrial coupling with mitochondrial biogenesis also plays a dual role in cancer. In this min-review, we discussed the context-dependent role of mitophagy in pancreatic cancer. Although this information enhances our understanding of the role of mitochondrial homeostasis in pancreatic cancer, there are still some key questions about the process and function of mitophagy in PDAC. How does the multi-step mitophagy actually proceed at different stages of PDAC? What are the key molecules or signals that distinguish the functions of mitophagy in promoting or inhibiting pancreatic tumorigenesis? Do tumor cells and non-tumor cells (such as immune cells or stromal cells) in the pancreatic tumor microenvironment have different mitophagy activities? In the pancreatic tumor microenvironment, what is the synergy or competition between mitophagy and other types of selective autophagy? How to develop specific mitophagy targeted drugs to kill pancreatic tumors? Are there specific markers to assess the level of mitophagy in PDAC patients?

AUTHOR CONTRIBUTIONS

YX and DT conceived of the topic for this review. All authors contributed to the article and approved the submitted version.

FUNDING

YX was supported by the National Natural Science Foundation of China (No. 81802476).

REFERENCES

1. Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res* (2014) 74:2913–21. doi: 10.1158/0008-5472.CAN-14-0155
2. Amanam I, Chung V. Targeted Therapies for Pancreatic Cancer. *Cancers (Basel)* (2018) 2:36. doi: 10.3390/cancers10020036
3. Levine B, Kroemer G. Biological Functions of Autophagy Genes: A Disease Perspective. *Cell* (2019) 176:11–42. doi: 10.1016/j.cell.2018.09.048
4. Dikic I, Elazar Z. Mechanism and medical implications of mammalian autophagy. *Nat Rev Mol Cell Biol* (2018) 19:349–64. doi: 10.1038/s41580-018-0003-4
5. Bandyopadhyay U, Kaushik S, Varticovski L, Cuervo AM. The chaperone-mediated autophagy receptor organizes in dynamic protein complexes at the lysosomal membrane. *Mol Cell Biol* (2008) 28:5747–63. doi: 10.1128/MCB.02070-07
6. Chiang HL, Terlecky SR, Plant CP, Dice JF. A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. *Science* (1989) 246:382–5. doi: 10.1126/science.2799391
7. Cuervo AM, Dice JF. A receptor for the selective uptake and degradation of proteins by lysosomes. *Science* (1996) 273:501–3. doi: 10.1126/science.273.5274.501
8. Xie Y, Kang R, Sun X, Zhong M, Huang J, Klionsky DJ, et al. Posttranslational modification of autophagy-related proteins in macroautophagy. *Autophagy* (2015) 11:28–45. doi: 10.4161/15548627.2014.984267
9. Liu J, Kuang F, Kroemer G, Klionsky DJ, Kang R, Tang D. Autophagy-Dependent Ferroptosis: Machinery and Regulation. *Cell Chem Biol* (2020) 27:420–35. doi: 10.1016/j.chembiol.2020.02.005
10. Green DR, Levine B. To be or not to be? How selective autophagy and cell death govern cell fate. *Cell* (2014) 157:65–75. doi: 10.1016/j.cell.2014.02.049
11. Vernon PJ, Tang D. Eat-me: autophagy, phagocytosis, and reactive oxygen species signaling. *Antioxid Redox Signal* (2013) 18:677–91. doi: 10.1089/ars.2012.4810
12. Gatica D, Lahiri V, Klionsky DJ. Cargo recognition and degradation by selective autophagy. *Nat Cell Biol* (2018) 20:233–42. doi: 10.1038/s41556-018-0037-z
13. Sharma V, Verma S, Seranova E, Sarkar S, Kumar D. Selective Autophagy and Xenophagy in Infection and Disease. *Front Cell Dev Biol* (2018) 6:147. doi: 10.3389/fcell.2018.00147
14. Yang M, Chen P, Liu J, Zhu S, Kroemer G, Klionsky DJ, et al. Clockophagy is a novel selective autophagy process favoring ferroptosis. *Sci Adv* (2019) 5:eaaw2238. doi: 10.1126/sciadv.aaw2238
15. Liu J, Yang M, Kang R, Klionsky DJ, Tang D. Autophagic degradation of the circadian clock regulator promotes ferroptosis. *Autophagy* (2019) 15:2033–5. doi: 10.1080/15548627.2019.1659623
16. Palikaras K, Lionaki E, Tavernarakis N. Mechanisms of mitophagy in cellular homeostasis, physiology and pathology. *Nat Cell Biol* (2018) 20:1013–22. doi: 10.1038/s41556-018-0176-2

17. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* (2000) 19:5720–8. doi: 10.1093/emboj/19.21.5720
18. Katsuragi Y, Ichimura Y, Komatsu M. p62/SQSTM1 functions as a signaling hub and an autophagy adaptor. *FEBS J* (2015) 282:4672–8. doi: 10.1111/febs.13540
19. Liu WJ, Ye L, Huang WF, Guo LJ, Xu ZG, Wu HL, et al. p62 links the autophagy pathway and the ubiqutin-proteasome system upon ubiquitinated protein degradation. *Cell Mol Biol Lett* (2016) 21:29. doi: 10.1186/s11658-016-0031-z
20. Xie Y, Li J, Kang R, Tang D. Interplay Between Lipid Metabolism and Autophagy. *Front Cell Dev Biol* (2020) 8:431. doi: 10.3389/fcell.2020.00431
21. Kang R, Zeng L, Xie Y, Yan Z, Zhou B, Cao L, et al. A novel PINK1- and PARK2-dependent protective neuroimmune pathway in lethal sepsis. *Autophagy* (2016) 12:2374–85. doi: 10.1080/15548627.2016.1239678
22. Li C, Zhang Y, Cheng X, Yuan H, Zhu S, Liu J, et al. PINK1 and PARK2 Suppress Pancreatic Tumorigenesis through Control of Mitochondrial Iron-Mediated Immunometabolism. *Dev Cell* (2018) 46:441–455 e8. doi: 10.1016/j.devcel.2018.07.012
23. Sliter DA, Martinez J, Hao L, Chen X, Sun N, Fischer TD, et al. Parkin and PINK1 mitigate STING-induced inflammation. *Nature* (2018) 561:258–62. doi: 10.1038/s41586-018-0448-9
24. Harper JW, Ordureau A, Heo JM. Building and decoding ubiquitin chains for mitophagy. *Nat Rev Mol Cell Biol* (2018) 19:93–108. doi: 10.1038/nrm.2017.129
25. Geisler S, Holmstrom KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, et al. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol* (2010) 12:119–31. doi: 10.1038/ncb2012
26. Wong YC, Holzbaur EL. Optineurin is an autophagy receptor for damaged mitochondria in parkin-mediated mitophagy that is disrupted by an ALS-linked mutation. *Proc Natl Acad Sci U S A* (2014) 111:E4439–48. doi: 10.1073/pnas.1405752111
27. Gao F, Chen D, Si J, Hu Q, Qin Z, Fang M, et al. The mitochondrial protein BNIP3L is the substrate of PARK2 and mediates mitophagy in PINK1/PARK2 pathway. *Hum Mol Genet* (2015) 24:2528–38. doi: 10.1093/hmg/ddv017
28. Lazarou M, Sliter DA, Kane LA, Sarraf SA, Wang C, Burman JL, et al. The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy. *Nature* (2015) 524:309–14. doi: 10.1038/nature14893
29. Wang Y, Serricchio M, Jauregui M, Shanbhag R, Stoltz T, Di Paolo CT, et al. Deubiquitinating enzymes regulate PARK2-mediated mitophagy. *Autophagy* (2015) 11:595–606. doi: 10.1080/15548627.2015.1034408
30. Yun J, Puri R, Yang H, Lizzio MA, Wu C, Sheng ZH, et al. MUL1 acts in parallel to the PINK1/parkin pathway in regulating mitofusin and compensates for loss of PINK1/parkin. *Elife* (2014) 3:e01958. doi: 10.7554/eLife.01958
31. Szargel R, Shani V, Abd Elghani F, Mekies LN, Liani E, Rott R, et al. The PINK1, synphilin-1 and SIAH-1 complex constitutes a novel mitophagy pathway. *Hum Mol Genet* (2016) 25:3476–90. doi: 10.1093/hmg/ddw189
32. Orvedahl A, Sumpter RJr., Xiao G, Ng A, Zou Z, Tang Y, et al. Image-based genome-wide siRNA screen identifies selective autophagy factors. *Nature* (2011) 480:113–7. doi: 10.1038/nature10546
33. Fu M, St-Pierre P, Shankar J, Wang PT, Joshi B, Nabi IR. Regulation of mitophagy by the Gp78 E3 ubiquitin ligase. *Mol Biol Cell* (2013) 24:1153–62. doi: 10.1091/mbc.e12-08-0607
34. Xie Y, Liu J, Kang R, Tang D. Mitophagy Receptors in Tumor Biology. *Front Cell Dev Biol* (2020) 8:594203. doi: 10.3389/fcell.2020.594203
35. Sandoval H, Thiagarajan P, Dasgupta SK, Schumacher A, Prchal JT, Chen M, et al. Essential role for Nix in autophagic maturation of erythroid cells. *Nature* (2008) 454:232–5. doi: 10.1038/nature07006
36. Liu L, Feng D, Chen G, Chen M, Zheng Q, Song P, et al. Mitochondrial outer-membrane protein FUNDC1 mediates hypoxia-induced mitophagy in mammalian cells. *Nat Cell Biol* (2012) 14:177–85. doi: 10.1038/ncb2422
37. O'Sullivan TE, Johnson LR, Kang HH, Sun JC. BNIP3- and BNIP3L-Mediated Mitophagy Promotes the Generation of Natural Killer Cell Memory. *Immunity* (2015) 43:331–42. doi: 10.1016/j.immuni.2015.07.012
38. Princely Abudu Y, Pankiv S, Mathai BJ, Hakon Lystad A, Bindesboll C, Brenne HB, et al. NIPSNAP1 and NIPSNAP2 Act as "Eat Me" Signals for Mitophagy. *Dev Cell* (2019) 49:509–525 e12. doi: 10.1016/j.devcel.2019.03.013
39. Wei Y, Chiang WC, Sumpter RJr., Mishra P, Levine B. Prohibitin 2 Is an Inner Mitochondrial Membrane Mitophagy Receptor. *Cell* (2017) 168:224–238 e10. doi: 10.1016/j.cell.2016.11.042
40. Murakawa T, Yamaguchi O, Hashimoto A, Hikoso S, Takeda T, Oka T, et al. Bcl-2-like protein 13 is a mammalian Atg32 homologue that mediates mitophagy and mitochondrial fragmentation. *Nat Commun* (2015) 6:7527. doi: 10.1038/ncomms8527
41. Bhujabal Z, Birgisdottir AB, Sjottem E, Brenne HB, Overvatn A, Habisov S, et al. FKBP8 recruits LC3A to mediate Parkin-independent mitophagy. *EMBO Rep* (2017) 18:947–61. doi: 10.15252/embr.201643147
42. Chu CT, Ji J, Dagda RK, Jiang JF, Tyurina YY, Kapralov AA, et al. Cardiolipin externalization to the outer mitochondrial membrane acts as an elimination signal for mitophagy in neuronal cells. *Nat Cell Biol* (2013) 15:1197–205. doi: 10.1038/ncb2837
43. Anderson GR, Wardell SE, Cakir M, Yip C, Ahn YR, Ali M, et al. Dysregulation of mitochondrial dynamics proteins are a targetable feature of human tumors. *Nat Commun* (2018) 9:1677. doi: 10.1038/s41467-018-04033-x
44. Yu M, Nguyen ND, Huang Y, Lin D, Fujimoto TN, Molkentine JM, et al. Mitochondrial fusion exploits a therapeutic vulnerability of pancreatic cancer. *JCI Insight* (2019) 4:e126915. doi: 10.1172/jci.insight.126915
45. Dai S, Peng Y, Zhu Y, Xu D, Zhu F, Xu W, et al. Glycolysis promotes the progression of pancreatic cancer and reduces cancer cell sensitivity to gemcitabine. *BioMed Pharmacother* (2020) 121:109521. doi: 10.1016/j.biopha.2019.109521
46. Daskalakis K, Alexandraki KI, Kloukina I, Kassi E, Felekouras E, Xingi E, et al. Increased autophagy/mitophagy levels in primary tumours of patients with pancreatic neuroendocrine neoplasms. *Endocrine* (2020) 68:438–47. doi: 10.1007/s12020-020-02228-1
47. Ko YH, Cho YS, Won HS, Jeon EK, An HJ, Hong SU, et al. Prognostic significance of autophagy-related protein expression in resected pancreatic ductal adenocarcinoma. *Pancreas* (2013) 42:829–35. doi: 10.1097/MPA.0b013e318279d0dc
48. Li J, Chen X, Kang R, Zeh H, Klionsky DJ, Tang D. Regulation and function of autophagy in pancreatic cancer. *Autophagy* (2020), 1–22. doi: 10.1080/15548627.2020.1847462
49. Levy JMM, Towers CG, Thorburn A. Targeting autophagy in cancer. *Nat Rev Cancer* (2017) 17:528–42. doi: 10.1038/nrc.2017.53
50. Monkkonen T, Debnath J. Inflammatory signaling cascades and autophagy in cancer. *Autophagy* (2018) 14:190–8. doi: 10.1080/15548627.2017.1345412
51. New M, Tooze S. The Role of Autophagy in Pancreatic Cancer-Recent Advances. *Biol (Basel)* (2019) 9:7. doi: 10.3390/biology9010007
52. Piffoux M, Eriau E, Cassier PA. Autophagy as a therapeutic target in pancreatic cancer. *Br J Cancer* (2021) 1244:333–44. doi: 10.1038/s41416-020-01039-5
53. Gorgulu K, Diakopoulos KN, Kaya-Aksoy E, Ciecielski KJ, Ai J, Lesina M, et al. The Role of Autophagy in Pancreatic Cancer: From Bench to the Dark Bedside. *Cells* (2020) 9:1063. doi: 10.3390/cells9041063
54. Kang R, Tang D. Autophagy in pancreatic cancer pathogenesis and treatment. *Am J Cancer Res* (2012) 2:383–96.
55. S NK, Wilson GW, Grant RC, Seto M, O'Kane G, Vajpeyi R, et al. Morphological classification of pancreatic ductal adenocarcinoma that predicts molecular subtypes and correlates with clinical outcome. *Gut* (2020) 69:317–28. doi: 10.1136/gutjnl-2019-318217
56. Yang S, Imamura Y, Jenkins RW, Canadas I, Kitajima S, Aref A, et al. Autophagy Inhibition Dysregulates TBK1 Signaling and Promotes Pancreatic Inflammation. *Cancer Immunol Res* (2016) 4:520–30. doi: 10.1158/2326-6066.CIR-15-0235
57. Loncle C, Molejon MI, Lac S, Tellechea JI, Lomberk G, Gramatica L, et al. The pancreatitis-associated protein VMP1, a key regulator of inducible autophagy, promotes Kras(G12D)-mediated pancreatic cancer initiation. *Cell Death Dis* (2016) 7:e2295. doi: 10.1038/cddis.2016.202
58. Rosenfeldt MT, O'Prey J, Morton JP, Nixon C, MacKay G, Mrowinska A, et al. p53 status determines the role of autophagy in pancreatic tumour development. *Nature* (2013) 504:296–300. doi: 10.1038/nature12865

59. Yamamoto K, Venida A, Yano J, Biancur DE, Kakiuchi M, Gupta S, et al. Autophagy promotes immune evasion of pancreatic cancer by degrading MHC-I. *Nature* (2020) 581:100–5. doi: 10.1038/s41586-020-2229-5
60. Kang R, Loux T, Tang D, Schapiro NE, Vernon P, Livesey KM, et al. 3rd, The expression of the receptor for advanced glycation endproducts (RAGE) is permissive for early pancreatic neoplasia. *Proc Natl Acad Sci U.S.A.* (2012) 109:7031–6. doi: 10.1073/pnas.1113865109
61. Waters AM, Der CJ. KRAS: The Critical Driver and Therapeutic Target for Pancreatic Cancer. *Cold Spring Harb Perspect Med* (2018) 8:a031435. doi: 10.1101/cshperspect.a031435
62. Lin HH, Chung Y, Cheng CT, Ouyang C, Fu Y, Kuo CY, et al. Autophagic reliance promotes metabolic reprogramming in oncogenic KRAS-driven tumorigenesis. *Autophagy* (2018) 14:1481–98. doi: 10.1080/15548627.2018.1450708
63. Nagdas S, Kashatus JA, Nascimento A, Hussain SS, Trainor RE, Pollock SR, et al. Drp1 Promotes KRas-Driven Metabolic Changes to Drive Pancreatic Tumor Growth. *Cell Rep* (2019) 28:1845–1859 e5. doi: 10.1016/j.celrep.2019.07.031
64. Palorini R, De Rasmo D, Gaviraghi M, Sala Danna L, Signorile A, Cirulli C, et al. Oncogenic K-ras expression is associated with derangement of the cAMP/PKA pathway and forskolin-reversible alterations of mitochondrial dynamics and respiration. *Oncogene* (2013) 32:352–62. doi: 10.1038/onc.2012.50
65. Meng N, Glorieux C, Zhang Y, Liang L, Zeng P, Lu W, et al. Oncogenic K-ras Induces Mitochondrial OPA3 Expression to Promote Energy Metabolism in Pancreatic Cancer Cells. *Cancers (Basel)* (2020) 12:65. doi: 10.3390/cancers12010065
66. Guo JY, Karsli-Uzunbas G, Mathew R, Aisner SC, Kamphorst JJ, Strohecker AM, et al. Autophagy suppresses progression of K-ras-induced lung tumors to oncocytomas and maintains lipid homeostasis. *Genes Dev* (2013) 27:1447–61. doi: 10.1101/gad.219642.113
67. Hingorani SR, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA, et al. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell* (2003) 4:437–50. doi: 10.1016/S1535-6108(03)00309-X
68. Humpton TJ, Alagesan B, DeNicola GM, Lu D, Yordanov GN, Leonhardt CS, et al. Oncogenic KRAS Induces NIX-Mediated Mitophagy to Promote Pancreatic Cancer. *Cancer Discovery* (2019) 9:1268–87. doi: 10.1158/2159-8290.CD-18-1409
69. Kang R, Xie Y, Zhang Q, Hou W, Jiang Q, Zhu S, et al. Intracellular HMGB1 as a novel tumor suppressor of pancreatic cancer. *Cell Res* (2017) 27:916–32. doi: 10.1038/cr.2017.51
70. Hingorani SR, Wang L, Multani AS, Combs C, Deramaudt TB, Hruban RH, et al. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell* (2005) 7:469–83. doi: 10.1016/j.ccr.2005.04.023
71. Tang D, Kang R, Livesey KM, Kroemer G, Billiar TR, Van Houten B, et al. High-mobility group box 1 is essential for mitochondrial quality control. *Cell Metab* (2011) 13:701–11. doi: 10.1016/j.cmet.2011.04.008
72. Tang D, Kang R, Livesey KM, Cheh CW, Farkas A, Loughran P, et al. Endogenous HMGB1 regulates autophagy. *J Cell Biol* (2010) 190:881–92. doi: 10.1083/jcb.200911078
73. Livesey KM, Kang R, Vernon P, Buchser W, Loughran P, Watkins SC, et al. p53/HMGB1 complexes regulate autophagy and apoptosis. *Cancer Res* (2012) 72:1996–2005. doi: 10.1158/0008-5472.CAN-11-2291
74. Chang HW, Kim MR, Lee HJ, Lee HM, Kim GC, Lee YS, et al. p53/BNIP3-dependent mitophagy limits glycolytic shift in radioresistant cancer. *Oncogene* (2019) 38:3729–42. doi: 10.1038/s41388-019-0697-6
75. Ito H, Fujita K, Tagawa K, Chen X, Homma H, Sasabe T, et al. HMGB1 facilitates repair of mitochondrial DNA damage and extends the lifespan of mutant ataxin-1 knock-in mice. *EMBO Mol Med* (2015) 7:78–101. doi: 10.15252/emmm.201404392
76. Li X, Deng S, Liu M, Jin Y, Zhu S, Deng S, et al. The responsively decreased PKM2 facilitates the survival of pancreatic cancer cells in hypoglucose. *Cell Death Dis* (2018) 9:133. doi: 10.1038/s41419-017-0158-5
77. Lee JS, Oh SJ, Choi HJ, Kang JH, Lee SH, Ha JS, et al. ATP Production Relies on Fatty Acid Oxidation Rather than Glycolysis in Pancreatic Ductal Adenocarcinoma. *Cancers (Basel)* (2020) 12:2477. doi: 10.3390/cancers12092477
78. Maan M, Peters JM, Dutta M, Patterson AD. Lipid metabolism and lipophagy in cancer. *Biochem Biophys Res Commun* (2018) 504:582–9. doi: 10.1016/j.bbrc.2018.02.097
79. Seo JW, Choi J, Lee SY, Sung S, Yoo HJ, Kang MJ, et al. Autophagy is required for PDAC glutamine metabolism. *Sci Rep* (2016) 6:37594. doi: 10.1038/srep37594
80. Kang R, Xie Y, Zeh HJ, Klionsky DJ, Tang D. Mitochondrial quality control mediated by PINK1 and PRKN: links to iron metabolism and tumor immunity. *Autophagy* (2019) 15:172–3. doi: 10.1080/15548627.2018.1526611
81. Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med* (2010) 49:1603–16. doi: 10.1016/j.freeradbiomed.2010.09.006
82. Seifert L, Werba G, Tiwari S, Giao Ly NN, Alothman S, Alqunaibit D, et al. The necrosome promotes pancreatic oncogenesis via CXCL1 and Mincle-induced immune suppression. *Nature* (2016) 532:245–9. doi: 10.1038/nature17403
83. Dai E, Han L, Liu J, Xie Y, Zeh HJ, Kang R, et al. Ferroptotic damage promotes pancreatic tumorigenesis through a TMEM173/STING-dependent DNA sensor pathway. *Nat Commun* (2020) 11:6339. doi: 10.1038/s41467-020-20154-8
84. Dai E, Han L, Liu J, Xie Y, Kroemer G, Klionsky DJ, et al. Autophagy-dependent ferroptosis drives tumor-associated macrophage polarization via release and uptake of oncogenic KRAS protein. *Autophagy* (2020) 16:2069–83. doi: 10.1080/15548627.2020.1714209
85. Tang D, Kang R, Berghe TV, Vandenabeele P, Kroemer G. The molecular machinery of regulated cell death. *Cell Res* (2019) 29:347–64. doi: 10.1038/s41422-019-0164-5
86. Bialik S, Dasari SK, Kimchi A. Autophagy-dependent cell death - where, how and why a cell eats itself to death. *J Cell Sci* (2018) 131:jcs215152. doi: 10.1242/jcs.215152
87. Zhou B, Liu J, Kang R, Klionsky DJ, Kroemer G, Tang D. Ferroptosis is a type of autophagy-dependent cell death. *Semin Cancer Biol* (2020) 66:89–100. doi: 10.1016/j.semcancer.2019.03.002
88. Guerra F, Guaragnella N, Arbini AA, Bucci C, Giannattasio S, Moro L. Mitochondrial Dysfunction: A Novel Potential Driver of Epithelial-to-Mesenchymal Transition in Cancer. *Front Oncol* (2017) 7:295. doi: 10.3389/fonc.2017.00295
89. Alcala S, Sancho P, Martinelli P, Navarro D, Pedrero C, Martin-Hijano L, et al. ISG15 and ISGylation is required for pancreatic cancer stem cell mitophagy and metabolic plasticity. *Nat Commun* (2020) 11:2682. doi: 10.1038/s41467-020-16395-2
90. Galluzzi L, Vitale I, Aaronson SA, Abrams JM, Adam D, Agostinis P, et al. Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death Differ* (2018) 25:486–541. doi: 10.1038/s41418-018-0102-y
91. Huang J, Chen P, Liu K, Liu J, Zhou B, Wu R, et al. CDK1/2/5 inhibition overcomes IFNG-mediated adaptive immune resistance in pancreatic cancer. *Gut* (2020). doi: 10.1136/gutjnl-2019-320441
92. Arlt A, Muerkoster SS, Schafer H. Targeting apoptosis pathways in pancreatic cancer. *Cancer Lett* (2013) 332:346–58. doi: 10.1016/j.canlet.2010.10.015
93. Xie Y, Zhu S, Zhong M, Yang M, Sun X, Liu J, et al. Inhibition of Aurora Kinase A Induces Necroptosis in Pancreatic Carcinoma. *Gastroenterology* (2017) 153:1429–1443 e5. doi: 10.1053/j.gastro.2017.07.036
94. Huang C, Lan W, Fraunhoffer N, Meilerman A, Iovanna J, Santofimia-Castano P. Dissecting the Anticancer Mechanism of Trifluoperazine on Pancreatic Ductal Adenocarcinoma. *Cancers (Basel)* (2019) 11:1869. doi: 10.3390/cancers11121869
95. Song X, Zhu S, Xie Y, Liu J, Sun L, Zeng D, et al. JTC801 Induces pH-dependent Death Specifically in Cancer Cells and Slows Growth of Tumors in Mice. *Gastroenterology* (2018) 154:1480–93. doi: 10.1053/j.gastro.2017.12.004
96. Liu J, Kuang F, Kang R, Tang D. Alkaliptosis: a new weapon for cancer therapy. *Cancer Gene Ther* (2020) 27:267–9. doi: 10.1038/s41417-019-0134-6

97. Li C, Zhang Y, Liu J, Kang R, Klionsky DJ, Tang D. Mitochondrial DNA stress triggers autophagy-dependent ferroptotic death. *Autophagy* (2020), 1–13. doi: 10.1080/15548627.2020.1739447
98. Zhu S, Zhang Q, Sun X, Zeh HJ3rd3rd, Lotze MT, Kang R, et al. HSPA5 Regulates Ferroptotic Cell Death in Cancer Cells. *Cancer Res* (2017) 77:2064–77. doi: 10.1158/0008-5472.CAN-16-1979
99. Xie Y, Kuang F, Liu J, Tang D, Kang R. DUSP1 Blocks Autophagy-Dependent Ferroptosis in Pancreatic Cancer. *J Pancreatol* (2020) 3:154–60. doi: 10.1097/JP9.0000000000000054
100. Badgley MA, Kremer DM, Maurer HC, DelGiorno KE, Lee HJ, Purohit V, et al. Cysteine depletion induces pancreatic tumor ferroptosis in mice. *Science* (2020) 368:85–9. doi: 10.1126/science.aaw9872
101. Hou W, Xie Y, Song X, Sun X, Lotze MT, Zeh HJ3rd3rd, et al. Autophagy promotes ferroptosis by degradation of ferritin. *Autophagy* (2016) 12:1425–8. doi: 10.1080/15548627.2016.1187366
102. Bock FJ, Tait SWG. Mitochondria as multifaceted regulators of cell death. *Nat Rev Mol Cell Biol* (2020) 21:85–100. doi: 10.1038/s41580-019-0173-8
103. Zhao C, He R, Shen M, Zhu F, Wang M, Liu Y, et al. PINK1/Parkin-Mediated Mitophagy Regulation by Reactive Oxygen Species Alleviates Rocaglamide A-Induced Apoptosis in Pancreatic Cancer Cells. *Front Pharmacol* (2019) 10:968. doi: 10.3389/fphar.2019.00968
104. Hu Y, Wang B, Wang L, Wang Z, Jian Z, Deng L. Mammalian STE20like kinase 1 regulates pancreatic cancer cell survival and migration through Mfn2mediated mitophagy. *Mol Med Rep* (2020) 22:398–404. doi: 10.3892/mmr.2020.11098
105. Kshattry S, Saha A, Gries P, Tiziani S, Stone E, Georgiou G, et al. Enzyme-mediated depletion of l-cyst(e)ine synergizes with thioredoxin reductase inhibition for suppression of pancreatic tumor growth. *NPJ Precis Oncol* (2019) 3:16. doi: 10.1038/s41698-019-0088-z
106. Tataranni T, Agriesti F, Pacelli C, Ruggieri V, Laurenzana I, Mazzoccoli C, et al. Dichloroacetate Affects Mitochondrial Function and Stemness-Associated Properties in Pancreatic Cancer Cell Lines. *Cells* (2019) 8:478. doi: 10.3390/cells8050478
107. Jia S, Xu X, Zhou S, Chen Y, Ding G, Cao L. Fisetin induces autophagy in pancreatic cancer cells via endoplasmic reticulum stress- and mitochondrial stress-dependent pathways. *Cell Death Dis* (2019) 10:142. doi: 10.1038/s41419-019-1366-y
108. Liu YH, Weng YP, Tsai HY, Chen CJ, Lee DY, Hsieh CL, et al. Aqueous extracts of Paeonia suffruticosa modulates mitochondrial proteostasis by reactive oxygen species-induced endoplasmic reticulum stress in pancreatic cancer cells. *Phytomedicine* (2018) 46:184–92. doi: 10.1016/j.phymed.2018.03.037
109. Saraei P, Asadi I, Kakar MA, Moradi-Kor N. The beneficial effects of metformin on cancer prevention and therapy: a comprehensive review of recent advances. *Cancer Manag Res* (2019) 11:3295–313. doi: 10.2147/CMAR.S200059
110. Liang C, Xu J, Meng Q, Zhang B, Liu J, Hua J, et al. TGFB1-induced autophagy affects the pattern of pancreatic cancer progression in distinct ways depending on SMAD4 status. *Autophagy* (2020) 16:486–500. doi: 10.1080/15548627.2019.1628540
111. Yang A, Herter-Sprie G, Zhang H, Lin EY, Biancur D, Wang X, et al. Autophagy Sustains Pancreatic Cancer Growth through Both Cell-Autonomous and Nonautonomous Mechanisms. *Cancer Discovery* (2018) 8:276–87. doi: 10.1158/2159-8290.CD-17-0952
112. Bryant KL, Stalnecker CA, Zeitouni D, Klomp JE, Peng S, Tikunov AP, et al. Combination of ERK and autophagy inhibition as a treatment approach for pancreatic cancer. *Nat Med* (2019) 25:628–40. doi: 10.1038/s41591-019-0368-8

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Xie, Liu, Kang and Tang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Oncology

REVIEW
published: 08 March 2021
doi: 10.3389/fonc.2021.603224

Autophagy in Viral Development and Progression of Cancer

Alejandra Suares[1,2], *María Victoria Medina*[1,2] *and Omar Coso*[1,2]*

[1] Departamento de Fisiología y Biología Molecular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina, [2] Instituto de Fisiología, Biología Molecular y Neurociencias (IFIBYNE), CONICET—Universidad de Buenos Aires, Buenos Aires, Argentina

Autophagy is a complex degradative process by which eukaryotic cells capture cytoplasmic components for subsequent degradation through lysosomal hydrolases. Although this catabolic process can be triggered by a great variety of stimuli, action in cells varies according to cellular context. Autophagy has been previously linked to disease development modulation, including cancer. Autophagy helps suppress cancer cell advancement in tumor transformation early stages, while promoting proliferation and metastasis in advanced settings. Oncoviruses are a particular type of virus that directly contribute to cell transformation and tumor development. Extensive molecular studies have revealed complex ways in which autophagy can suppress or improve oncovirus fitness while still regulating viral replication and determining host cell fate. This review includes recent advances in autophagic cellular function and emphasizes its antagonistic role in cancer cells.

Keywords: autophagy, human diseases, cancer cells, oncoviruses, cell survival, cell death

OPEN ACCESS

Edited by:
Daniel Hector Grasso, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

Reviewed by:
Alfredo Criollo, University of Chile, Chile
Priyanka Gupta, University of Alabama at Birmingham, United States

***Correspondence:**
Omar Coso
ocoso@fbmc.fcen.uba.ar

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *05 September 2020*
Accepted: *12 January 2021*
Published: *08 March 2021*

Citation:
Suares A, Medina MV and Coso O (2021) Autophagy in Viral Development and Progression of Cancer. Front. Oncol. 11:603224. doi: 10.3389/fonc.2021.603224

INTRODUCTION

Living organisms survive and are naturally preserved thanks to the combination of complex systems that coordinate to maintain homeostatic balance (1). The immune and endocrine systems represent good examples, as specialized cells and chemical mediators work together with antibodies and hormones to generate a specific response in the body (2). Individuals constantly face tissue damage due to stressful and environmental signals, as well as normal body deterioration and aging consequences (3). This is why organisms need intracellular signaling mechanisms that allow them to protect themselves from damaged cells, either by killing them or inhibiting their spread (4). In this way, organisms are prevented from preserving defective cell lines with potential mutation or error accumulation that may contribute to disease risk (5).

Macroautophagy (autophagy) is a metabolic process of intracellular component autodegradation, such as proteins and organelles, crucial for maintaining metabolism and cellular homeostasis (6). Normal levels of basal autophagy prevent cells from gradually accumulating proteins and damaged organelles that can become toxic to cells over time (7). Identifying the mechanistic components of this process at the cellular and molecular levels has been of great interest to researchers worldwide since the late 1950s (8). The first scientists to study and coin the name this catabolic mechanism believed that autophagy was just a cytoplasmic "*cleaning mechanism*" by which cells remove harmful components that accumulate in the cytoplasm (9). This explains the etymology of the term, which comes from the Greek words "*phagy*," meaning "*eat*," and

"*auto*," meaning "*me*." However, the role of autophagy in cells is now considered to be much broader as well as strongly influenced by the cellular environment. Autophagy modulation is related to human pathophysiology, and its implications affect different medical fields (10). This review summarizes the advances in molecular biology in relation to how this catabolic process helps develop different human diseases, focusing primarily on autophagy's dual role in health maintenance and tumor progression, with special interest in tumors associated with viral infections.

MECHANISM OF AUTOPHAGY

Up to date, 32 *atg* (autophagy-related genes), involved in regulating different autophagy stages have been identified in mammals. These genes encode numerous proteins (ATG) that regulate the autophagic machinery (11). Autophagy can be divided mechanistically into different stages: 1) initiation and nucleation (molecule recruitment for isolation membrane extension), 2) phagophore elongation and closure (autophagosome), 3) fusion with lysosomes (autolysosomes), 4) degradation, and 5) cytoplasmic material recycling (12) (**Figure 1**). Mammalian cells induce the autophagic machinery in response to various cellular stimuli, such as prolonged starvation (13), decreased glucose levels (14), hypoxia (15–17), increased levels of reactive oxygen species (ROS) (18, 19), and ER (endoplasmic reticulum) stress (20, 21), among others (22).

AMPK protein (AMP-activated kinase) is the main inducer of the autophagic machinery thus reducing intracellular ATP levels. Recent studies have revealed AMPK's fundamental role in autophagosome maturation and its fusion with lysosomes (23). Similarly, mTOR (mammalian target of rapamycin) activity is regulated by amino acid and glucose levels in mammalian cells (24), mTOR being their main autophagy inhibitor (25). Specifically, mTORC1 (mTOR complex 1) detects the cell's nutritional status and activates various signaling pathways to regulate cell fate (26). mTORC1 detects and responds to fluctuations of intra and extracellular nutrient levels, mainly amino acids and oxygen, as well as various growth factors (27). A complex dynamic between mTOR and AMPK enables coordinated regulation of signaling pathways in response to cellular environment changes (28).

High-nutrient levels promote mTORC1 inactivation and the induction of anabolic pathways involving protein, lipid, and nucleotide synthesis through S6K (ribosomal protein kinase S6) and 4E-BP1 (initiation factor of the eukaryotic translation 4E - binding protein 1) phosphorylation (29). At the same time, catabolic cellular programs are suppressed as ULK1 is inhibited (Unc-51-like kinase 1, mammalian homolog of *atg1*), thus leading -in turn- to autophagy inhibition (30). Multiprotein complex ULK1 (ULK1, Beclin-1 (BECN1), and PI3KCIII (phosphatidylinositol-3 kinase class III)) mainly regulates the autophagic mechanism initiation process (31). Once active, the complex is recruited to the isolation membrane, where it contributes to PIP3 (phosphatidylinositol 3-phosphate) formation and Akt activation (32). These cellular events dampen TSC 1/2 (tuberous sclerosis protein 1 and 2)inhibitory effect, a protein heterodimer homologous to RHEB (RAS

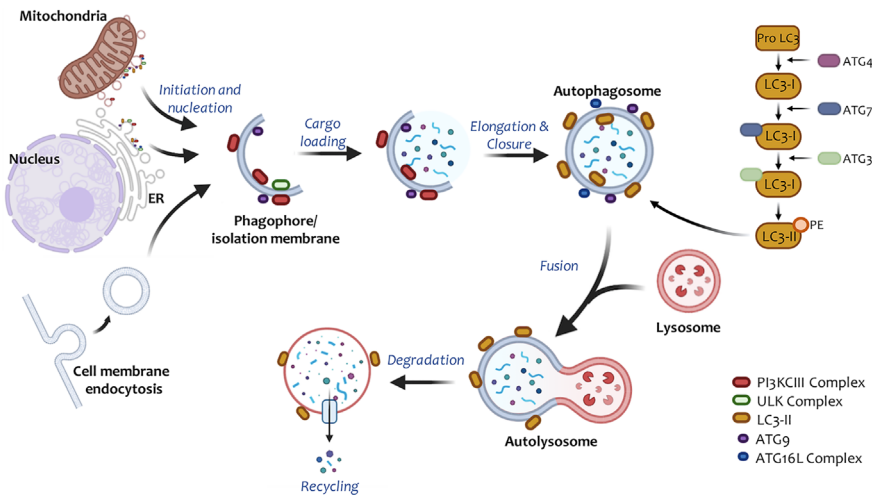


FIGURE 1 | Steps involved in autophagic responses. Autophagy begins with the progressive segregation of cytoplasmic material by double-membrane structures, commonly known as phagophores or isolation membranes. In general, this process is preceded by the inactivation of the PI3K/Akt/mTOR signaling axis. Phagophores are nucleated primarily from the endoplasmic reticulum (ER), but other organelles, such as the Golgi apparatus, plasma membrane, mitochondria, and recycling endosomes have also been shown to participate in this process. The complexes ULK1 and PI3KCIII are involved during phagophore initiation and elongation. Pro-LC3 is free in the cytoplasm and by the action of ATG4 and ATG7, LC3-I is formed. This molecule interacts with the complex ATG16L, ATG3, to later incorporate a phosphatidylethanolamine (PE) molecule into its structure. This results in LC3-II, which binds to autophagosomal membranes and contributes to phagophore elongation as well as closure. Then, these membranous structures seal, and autophagosomes are entirely assembled. Subsequently, autophagosomes fuse with lysosomes to form single membrane structures called autolysosomes, where the degradative process takes place through lysosomal hydrolases. The degradation products of these catabolic reactions reach the cytosol through transporters in the lysosomal membrane and are recycled by bioenergetic circuits.

enriched in brain protein) (33, 34). Akt can also be inhibited by mTORC2, further contributing to autophagy inhibition (35). PTEN (phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase) can hinder PIP3 formation, by activating the PI3K/Akt/mTOR pathway (36).

How the isolation membrane resulting in the phagophore forms still remains unclear. However, this membrane has been reported to derive from the plasma membrane, the endoplasmic reticulum (37), the Golgi apparatus (38), the ER-Golgi intermediate compartment (39), and the mitochondria (40). In these membranes, nucleation occurs (41) (**Figure 1**). All proteins involved in pagophore elongation, maturation, and closure are recruited through this process (42). The phagophore incorporates and degrades cytoplasmic material during extension and then it closes up, forming double-membrane vesicular structures called autophagosomes (35).

The action of PI3KCIII multiprotein complex (VPS34 (vacuolar proteins sorting 34), BECN1, p150 (ortholog of mammals of VPS15), mAtg14) in the protein recruitment process to the isolation membrane is fundamental (12). At this point, achieving active recycling is essential, a process involving the intervention of the ATG9 protein anchored in the membranes (31). Some Bcl-2 (B-cell lymphoma 2) family members, such as Bcl-2 and Bcl-xL (B-cell lymphoma extra-large), are known inhibitors of programmed cell death but can also inhibit autophagy through their interaction with BECN1 (43–45). Such interaction does not allow BECN1 to interact with VPS34 (46–48).

After nucleation, the ATG16L complex (ATG12, ATG5, ATG16L1) is recruited into the membrane, where it contributes to LC3 (light chain microtubule-associated protein-1 or MAP1LC3B) (49), GATE-16 (Golgi-associated ATPase enhancer of 16 kDa), and GABARAP (aminobutyric protein associated with the γ-acid receptor) lipidation (11, 50). These three groups belong to the ATG8 protein family, highly conserved across the evolutionary scale (51). Several proteins are anchored to the phagophore membrane, which is shed and returned to the cytoplasm before closure. Meanwhile, LC3 remains attached to the autophagosomal membranes throughout the process, making it a useful autophagosome marker (52) (**Figure 1**).

Several cellular receptors are involved in the selective recognition and recruitment of the cytoplasmic material that is later degraded in autolysosomes. The best-characterized autophagy receptor to date is p62 (Sequestosome 1 or SQSTM1), a molecular adapter with a ubiquitin-binding site and another for LC3 (35). p62 can also promote inflammatory gene expression through NF-kB (nuclear factor kB) regulation, activated when binding to TRAF6 (tumor necrosis factor receptor-associated factor 6) (53). Furthermore, p62 has been shown to activate an antioxidant response by sequestering Keap-1 (Kelch-like ECH-associated protein-1) through an Nrf2-dependent (erythroid-derived nuclear factor 2) mechanism (54) as well as to activate mTORC1 and regulate c-Myc (55).

After formation, autophagosomes relocalize to the perinuclear region through microtubules, where they fuse with lysosomes to form single membrane vesicles called autolysosomes (56), a complex process requiring anchoring factors and about which very little is known so far (57). SNARE proteins (soluble NSF binding protein) take part in the recognition and fusion of these structures. Studies in mice have revealed the importance of a SNARE complex [VTI1B, syntaxin 8, syntaxin 7, and VAMP-8 (vesicle-associated membrane protein 8)] in late fusion with the lysosome (Vadim Atlashkin2003). UVRAG (a gene associated with ultraviolet radiation) can activate GTPase RAB7 to promote fusion (58). Syntaxin 17, located in mature autophagosomes, can also regulate this mechanism (59). Successful binding to lysosomes is necessary for complete autophagy, as lysosomes provide the lytic enzymes needed for the degradation of cytoplasmic components in vesicles (60).

FUNCTIONS OF AUTOPHAGY

Historically, autophagy was considered to be a mechanism benefitting cell survival, as it recycles damaged and potentially toxic cytoplasmic components to increase vitality in cells subjected to stressful conditions, such as nutrient deprivation (61). Depending on cellular context, autophagy is selective or non-selective (massive autophagy). The former shows high specificity in degradation load selection and delivery, while in the latter, cytoplasmic particles are incorporated randomly (62). Selective autophagy is defined by the cytoplasmic material digested in mitophagy (affects mitochondria) (63), pexophagy (peroxisomes) (64), reticulophagy (endoplasmic reticulum and ribosomes) (65), nucleophagy (nucleus) (66), lipophagy (fat cells) (67), xenophagy (involves pathogens and other non-host entities) (68), and aggrephagy (damaged protein aggregates) (69).

With the advancement of knowledge, autophagy has been found not only to promote cell survival, but also to be induced in dying cells (70). The role of autophagy in death is, even today, a critical controversial point among researchers. While some scientists consider autophagy to been an independent death mechanism (autosis) (71), others argue that activation in dying cells occurs as a failure to rescue the cells from the stressful stimuli leading them to death in the first place (72, 73). In light of the growing number of physiological functions related to the autophagic mechanism, connections with numerous human pathologies have also been strengthened (74).

THE ROLE OF AUTOPHAGY IN CANCER

For cell transformation and tumor development to happen, several basic cellular alterations -referred to as *the hallmarks of cancer*- must occur (75, 76). Increasing evidence suggests a link between autophagy and cancer (77).

However, establishing the role of autophagy in cancer has proved problematic as it can both contribute to tumor promotion and inhibition, depending on cellular context and

disease stage (78). In the early stages of tumor transformation, autophagy can be activated to help cells mitigate mutations and damage their various components. But, once the transformation is complete, tumor cells can make use of the autophagic machinery to meet the high metabolic requirements of these uncontrolled dividing cells (79) (**Figure 2**).

Autophagy as a Tumor-Suppressing Mechanism

The most relevant findings related to autophagy and its role as a tumor suppressor come from studies on BECN1 (80). Mice with depletion of an allele for this gene showed a higher predisposition to different neoplasm spontaneous development (81). BECN1 has a BH3 domain, so it is not surprising that it can interact with various members of the Bcl-2 protein family and homologous viral proteins (82). Through these interactions, BECN1 can regulate autophagic and programmed cell death (83). Under normal conditions, Bcl-2 inhibits BECN1, whereas under stress conditions, they dissociate. This allows BECN1 to interact with VPS34 and modulate autophagy (84).

BECN1 dysregulation has been associated with the development of several cancers, including 50%–70% of prostate, breast, and ovarian cancers (85). In response to stressful stimuli, BECN1 can interact with Bif-1 (bax1 interaction factor or endophilin B1) through a mechanism involving UVRAG, thus leading to PI3KCIII and VPS34 activation. This regulatory mechanism has been evidenced in various tumor models (86).

Under cellular stress conditions, autophagy induction mitigates oxidative stress by eliminating damaged mitochondria, a crucial source in ROS production (80). When defective autophagy occurs, debris cannot be removed, leading to

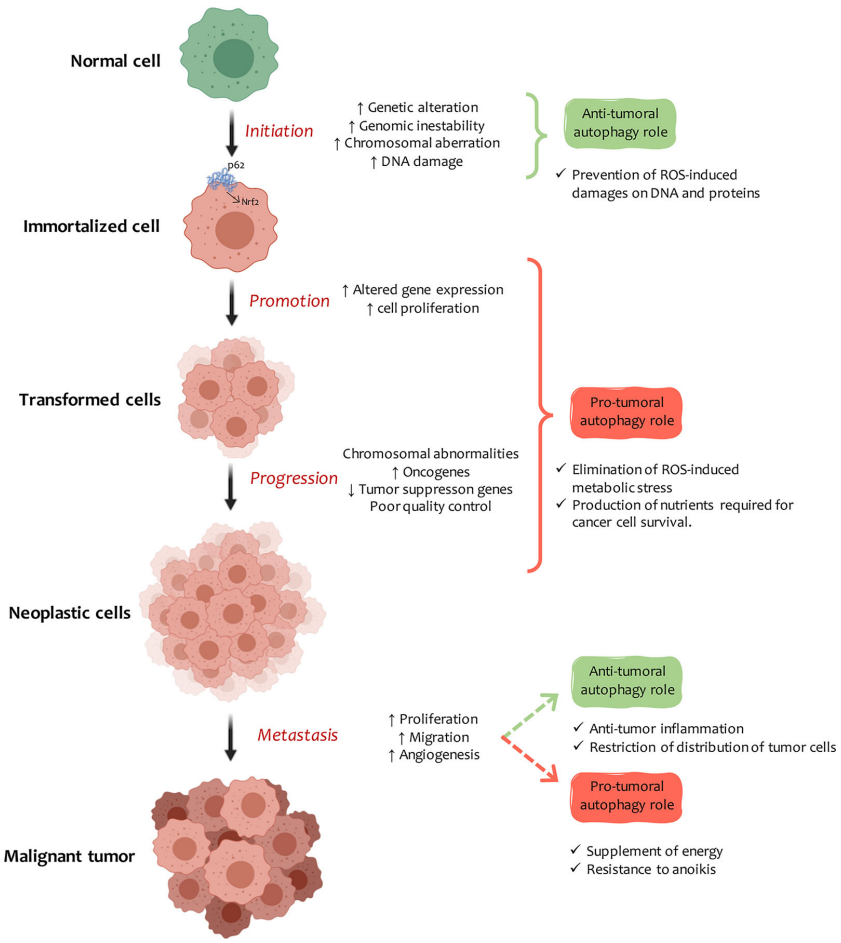


FIGURE 2 | Autophagy and cancer. Autophagy plays a dual role in the development of cancer, the nature of which depends on the tissue, stage, and type of tumor. In carcinogenesis early stage, autophagy induction protects cells from DNA and protein damage due to the indiscriminate increase in ROS and cell-toxic components. Once cells are immortalized, autophagy plays an essential role in promoting tumors. Autophagy induction can be modified, or at least some of the steps involved in the mechanism can be changed. The excessive accumulation of autophagic vesicles leads to p62 molecule accumulation and intracellular signaling activation, which leads to Nrf2 transformation, inflammation, and cell necrosis. In tumor progression, autophagy provides high-energy nutrients to actively dividing transformed cells. When transformed cells metastasize, autophagy can inhibit the process by promoting anti-tumor inflammatory responses or by restricting the expansion of dormant metastatic tumor cells. On the contrary, it supports metastasis by improving cell fitness against a stressful microenvironment (anoikis).

increased ROS levels and DNA damage with consequent loss of genomic integrity (87, 88). Therefore, autophagy prevents tumor generation by regulating ROS levels (89).

Atg genes expression has been directly linked to this process. Studies in mouse liver with silenced *atg7* and *atg5* showed autophagy malfunction as contributing to benign hepatic adenoma development (90). These tumors did not progress over time, suggesting that the loss of autophagy may be sufficient for tumorigenesis onset, but not for progression towards advanced stages of the disease (91). On the other hand, mice with deficient in *atg4C* showed alterations in autophagy and greater predisposition to fibrosarcomas induced by carcinogens (92).

Numerous tumor suppressor proteins promote autophagy (93–95). Tumor suppressor p53 is a usually deregulated protein in many human neoplasms which promotes autophagy when activated by nutrient deprivation or genotoxic stress (91). p53 functional loss is therefore expected to lead to autophagy inhibition (96). However, p53 can act as either an activator or an inhibitor of autophagy depending on its subcellular localization and its action mode (97). Mice with pancreatic oncogenic alleles for *k-ras* develop precancerous lesions and PDCA (pancreatic ductal adenocarcinoma) over time. Here, p53 expression blocks autophagy, thus inhibiting initial carcinogenesis (98). On the other hand, p53 can contribute to autophagy activation through DRAM1 (DNA damage-regulated autophagy modulator protein 1) (99), *atg7*, and *ulk1* (100) modulation. Another p53 target gene is *isg20l1*, which promotes autophagy induction and cell death when activated (101).

Numerous studies have revealed an increase in autophagy levels as carcinogenesis progresses (89). In metastasis early stages, cells acquire migratory properties and detach from the tumor to enter the bloodstream and flow through the body to colonize new tissue. At this point, autophagy exerts an anti-tumor role by modulating inflammation and cell shedding, but it also promotes motility and invasion (102). These results suggest that the autophagic machinery is a regulatory mechanism that can inhibit tumor generation in the early stages of the disease and in metastasis (**Figure 2**).

Autophagy as a Pro-Survival and Resistance Mechanism

High metabolism requires a stressful condition to which tumor cells must adapt to proliferate actively in combination with a hypoxic cellular environment (86). Under these conditions, cells can activate autophagy to address various cellular needs and promote oncogenesis (77). Autophagy is activated in the hypoxic regions of tumors to counteract cellular oxygen demand (103). When tumor cells blood supply is insufficient, the autophagic machinery can be activated through an HIF-1-dependent mechanism (hypoxia-inducible factor-1) (15), VEGF (vascular endothelial growth factor) (104), PDGF (platelet-derived growth factor) (105) and oxide synthase (106). Hence, autophagy plays an essential role in promoting tumor cell survival under metabolic stress (107). Furthermore, cell division high rate translates into increased energy and biosynthetic needs, which can be satisfied by rising autophagy levels to obtain ATP and metabolic intermediates (108).

Transcription factor p53 acts as a cellular stress sensor in response to DNA damage and oncogenic stress (94) and often mutates in different types of human cancers (109). Moreover, point mutations in p53 prevent it from inhibiting autophagy in some breast cancer models (96). Consequently, this catabolic signaling pathway is activated to help repair damaged DNA and benefit tumor cells (110).

p62 cell adapter is another crucial molecule in nutrient detection. It can also act as a mitotic transit modulator, an oxidative detoxifying protein inducer, and genomic stability regulator (111). It also contributes to the autophagic mechanism by recruiting proteins and organelles into the autophagosomal compartments for subsequent degradation (112). In liver carcinoma cells, mTORC1 inhibition and ER stress promote p62 accumulation and autophagy induction (113). In colorectal cancer cells, p62 promotes invasion and metastasis by inhibiting apoptosis through a mechanism involving the vitamin D receptor and Nrf2 (114). p62 deletion produces significant autophagy inhibition and affects tumor growth in *in vivo* and xenograft models (115). Upregulated p62 is commonly found in various tumor models (111).

Approximately 33% of neoplasms developed in mammals present mutations of the *ras* gene, which indicates the importance of this gene for medical science (116). Recent studies have revealed that autophagy promotes tumor development, invasion, and metastasis in epithelial cells transformed by this oncogene (117). Furthermore, autophagy inhibition in mice with lung tumors induced by *k-ras* expression was fatal, as specimens died of pneumonia (118). In pancreatic adenocarcinoma models with *k-ras* mutations, tumorigenicity was associated with increases in IL-1 (interleukin-1), NF-kB, and p62 levels (119).

Many patients manifest metastatic bodies many years after the primary tumor has appeared (120). Autophagy can suppress cell division and motility, thus conserving dormant tumor cells energy. At some point, latent cells can reactivate proliferation and colonize new tissue in response to changes in the tumor microenvironment (121). Anoikis is an apoptotic cell death model triggered by insufficient interaction between the cell and the extracellular matrix, a critical factor for transformed cell invasion and metastasis (122). When cells detach from their matrix to enter the bloodstream, autophagy protects them from anoikis and promotes metastasis (123). On the other hand, autophagy contributes to carcinogenesis by inhibiting apoptosis caused by mitochondrial dysfunction and excessive ROS production (124) (**Figure 2**).

AUTOPHAGY WITHIN THE TUMORAL MICROENVIRONMENT

The tumor microenvironment is the tumor stroma and occupies most of the neoplasm (125). We can therefore hypothesize that as carcinogenesis progresses, the interaction between tumor cells

and surrounding stromal cells increases (126). Compared to normal tissues, the tumor microenvironment is characterized by low oxygen levels, high lactate levels, extracellular acidosis, and decreased nutrients (127). It presents great cellular heterogeneity, composed of mesenchymal stem cells, fibroblasts, endothelial cells, immune cells, cytokines, and growth factors (128). Cancer-associated fibroblasts (CAF) are the most studied cells in the tumor microenvironment as they play an active role in tumor promotion (129). These components cooperate to contribute to tumor development (121) (**Figure 3**, top panel).

Autophagy is activated in the tumor microenvironment and in adjacent transformed cells to ensure tissue remodeling, angiogenesis, and interaction with surrounding immune cells (126). Autophagy has been detected in most cells of the tumor microenvironment, but with the ability of performing different actions. For example, when faced with a specific stimulus, the autophagic machinery in fibroblasts promotes tumorigenesis. Simultaneously, in some immune cells, such as cytotoxic T cells, it facilitates immune-response execution against neoplastic cells (130). Under extreme physiological conditions, the stroma activates autophagy to supply energy for adjacent tumor cells (131). In addition, tumor cells can modulate autophagic vesicles' induction in specific stromal cells (108).

For many years, cancer-related studies focused exclusively on transformed cells, ignoring the tumor environment. At present, the tumor microenvironment is considered to play a fundamental role in tumor development, and its study is

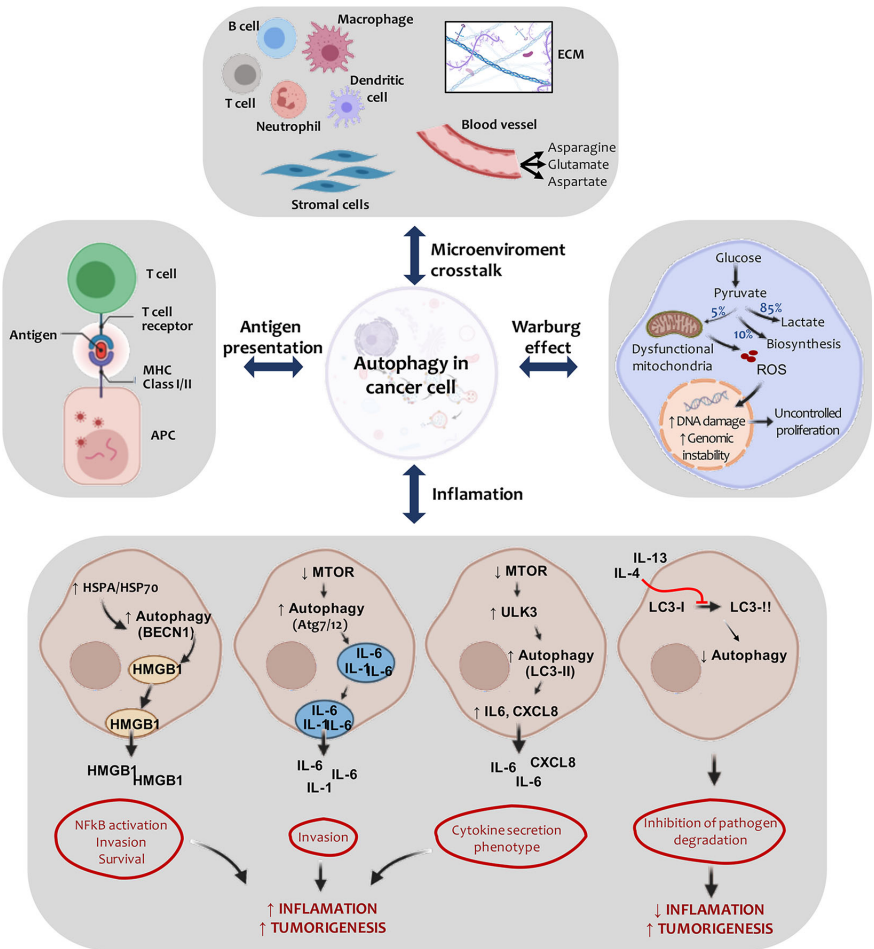


FIGURE 3 | Active crosstalk between autophagy and tumor microenvironment. Carcinogenesis is regulated by autophagy in transformed cells and cells belonging to the tumor microenvironment. Signaling is triggered to the ECM (extracellular matrix) and to stromal cells (such as fibroblasts and pericytes), generating a favorable context for tumor development. As tumor development progresses, cell autophagy activation in the tumor microenvironment serves, in part, to compensate for the inadequate nutritional supply associated with rapidly growing tumors. Here, proper quality control of the mitochondria is necessary to aid glycolysis in tumor cells. In this way, the energy balance (Warburg effect) typically found in malignant cells is maintained. Also, the autophagic process is involved in multiple aspects of lymphocyte development, innate immune signaling and antigen presentation by APCs (antigen presenting cells), processes that are relevant to the disease pathogenesis. HSPA/HSP70 overexpression can induce HMGB1 release in a BECN1-dependent process. This event culminates in the activation of NFκB and promotes tumor proliferation and invasion. Similarly, autophagy induction can contribute to IL-6, IL-1, and IL-8 secretion and promote inflammation. The expression of specific cytokines may inhibit this process, generating a decrease in the inflammatory process.

essential to form a cohesive idea of what happens within the tumor (132). The extracellular matrix is a fundamental component of the tumor microenvironment. In addition to providing a physical scaffold, it contributes to the secretion of key factors for the tumor's proper development. Macrophages and fibroblasts associated with cancer are the main cellular models associated with the tumor microenvironment (133).

The bone marrow is the leading site of hematopoiesis and bone formation in most vertebrates as well as the location of inactive and undifferentiated hematopoietic stem cells (HSC) (134). In response to specific stimuli, HSC can differentiate into various blood cells (135). As they have a short life cycle, HSC functional differentiation becomes necessary. During differentiation, cells are exposed to low oxygen levels, a sufficient condition for autophagy induction (136). Recent studies have revealed that autophagy is essential both for self-renewal and for HSC differentiation (137, 138).

After extravasation, monocytes are stimulated by CSF-1 (colony-stimulating factor-1) to induce differentiation. However, this stimulating factor is also associated with autophagy induction through ULK1 activation (139) and PI3K/Akt inhibition (140). CSF-2, another cytokine related to macrophage differentiation, prevents BECN1 and Bcl-2 interaction through a mechanism that includes JNK and triggers autophagy (141). At the same time, G-CSF (granulocyte colony-stimulating factor) contributes to cell survival through apoptosis inhibition and autophagy induction (142).

In recent years, studies related to the role of autophagy and CAFs in the tumor microenvironment have increased (143) and autophagic machinery induction has been associated with the NF-kB pathway and Caveolin-1 (Cav-1) expression in the tumor microenvironment (144, 145). Cav-1 is an essential component of the plasma membrane caveolar, contributing to modulating various signaling pathways. Other scientific sources have revealed a direct relationship between the autophagy induction and BNIP3 (E1B-binding protein 19K/Bcl-2 Nip3) expression in fibroblasts. This induction is accompanied by the loss of Cav-1 expression and the increase in BECN1 and ATG16L (146). Pancreatic stellate cells are a specialized type of fibroblast that can be found in tumor stroma, including PDAC (48, 147). Extensive studies have shown that stellate cells can secrete extracellular matrix molecules and cytokines that contribute to tumor aggressiveness (148). The baseline level of autophagy in PDAC patients is high, and active interaction between the tumor and stromal cell autophagy has been observed (149).

Tumor vasculature is involved in immune cell trafficking and activity. However, it also increases nutrient and oxygen circulation to meet solid tumors' high energy demands (150). A constant imbalance between pro-, and anti-angiogenic signaling in the tumor microenvironment exists, which contributes to new vessel formation through a VEGF-dependent mechanism (151, 152). Endothelial cells that reside in tumors are exposed to high VEGF levels, nutrient deprivation, and aberrant blood circulation, thus leading to increased autophagy levels (150). In fact, in tumor endothelial cells, autophagy levels are higher than in healthy endothelium (153). Said induction, as mentioned in the previous paragraph, can have anti-angiogenic (154) or pro-angiogenic (155) functions and, therefore, contribute to a different cell fate (156).

Autophagy as a Regulator of Tumor Immunity and Inflammation

Autophagy can induce immune system cells to exert specific responses (157). Recently, autophagy has been shown to influence not only the antigenic profile of antigen donor cells and their ability to release immunogenic signals (158, 159), but also the survival, differentiation, and function of antigen-presenting cells (APC) (160–162).

Innate immunity is the body's first line of defense against attack by pathogens; it is an active process and it favors the complement system as well as inflammation (163). At the cellular level, the presence of intracellular pathogens is detected by PRRs (pattern recognition receptors) located in the plasma membrane (TLR (Toll-like receptors), 1, 2, 4, 5, and 6), in endosomal membranes (TLR3, TLR7, TLR8, TLR9) or in the cytosol (NOD (Nod-type receptors), RIG-I (gene I-like receptors), RLR (retinoic acid-inducible receptors), and CLR (C-Type lectin-like receptors) (164). PRRs recognize surface antigens of microbes called PAMPs (pathogen-associated molecular patterns), such as lipopolysaccharides of the bacterial cell wall (LPS), flagellin, bacterial, and viral nucleic acids, and finally, some components of the fungal cell walls (165, 166).

After PPRs recognize pathogens, cells can induce autophagy to eliminate them through lysosomal degradation, although this is highly dependent on cellular context and cell type (167). Evidence has shown several TLRs, including TLR1, TLR3, TLR4, TLR5, TLR6, and TLR7, inducing autophagy in humans and mice macrophages (168). The connection between TLR signaling and autophagy is believed to be mediated by adapter proteins TRIF (adapter-inducing interferon-β containing the TIR domain) and Myd88 (Myeloid differentiation primary response 88), which inhibit the interaction between BECN1 and Bcl-2, thus contributing to autophagy induction (169). Furthermore, the link between Myd88 and mTOR has been reported to allow the activation of transcription factors (IRF-5 (interferon regulatory factor 5), IRF-7) that encode for pro-inflammatory cytokine genes and IFN-I (interferon type-I) (170).

Adaptive immunity, on the other hand, produces/makes a more robust and specific response (171), which involves capturing foreign material by APCs (macrophages, B cells, and dendritic cells) to stimulate T lymphocytes and give specific cellular responses (**Figure 3**, left panel) (172). APCs present antigens to major histocompatibility complex (MHC) molecules through the complex interaction of various cellular factors (173). Autophagy inhibition has been found to reduce MHC-I (MHC-class I) molecules in murine B16 melanoma cells and subsequent cytolysis of tumor cells by CD8+ T cells through cross-presentation (174). CD8+ T cells can respond to exogenous antigens and material undergoing phagocytosis (175). CD4 + T cells recognize antigens from MHC-II molecules (MHC-class II) that are processed in endolysosomal compartments (176).

Autophagy may be an essential source of MHC-II antigens derived from intracellular sources through lysosome material supply (177). Like T cells, B cells are regulated by the autophagic machinery. For example, deletion of *atg7* or *atg5* in the hematopoietic system results in a reduced number of peripheral B cells (178, 179). Dendritic cells are responsible for presenting pathogenic antigens to CD4+ and CD8+ lymphocytes, a process that is favored by autophagy induction (180).

Autophagy can regulate immunity and inflammation in tumor transformation in order to regulate carcinogenesis (181). Cytokine signaling is involved in tumor-associated inflammation and has been linked to promoting tumor-initiating cell self-renewal, tumor growth, angiogenesis, and metastasis (182). Cytokine secretion is variable depending on cancer type, but generally involves IL-1, IL-6, CXCL8/IL-8, IL-10, and interferon-gamma (183). In estrogen-receptor-negative breast tumors, IL-1 expression has been associated with autophagy induction with p62 and LC3 accumulation (184). In liver tissue carcinomas, IL-37 expression regulates autophagy by inhibiting the PI3K/Akt/mTOR axis (185). Some cytokines stimulate autophagy (Th1, TNF-α (tumoral necrosis factor α), IL-2), while others inhibit it (Th2, IL-4, IL-13, IL-10) (121) (**Figure 3**, bottom panel).

ROS accumulation in tumor development can cause mutations, protein and mitochondrial damage, and increased secretion of inflammatory and antimicrobial agents (186). An increase of intracellular ROS levels is commonly related to inflammatory signaling activation involving NF-kB and regulating the inflammatory response, angiogenesis, and the function of tumor-initiating cells, according to cellular context (187). Furthermore, interaction between NF-kB and the autophagic machinery in order to alter apoptosis and benefit tumor cell survival is common (181). Studies carried out in a murine model of lung adenocarcinoma have revealed that p62 deletion inhibits tumor development through a mechanism that prevents RELA/65 nuclear localization and NF-kB activation (188).

Autophagy in Tumor Cell Metabolism Remodeling

In the 1920s, scientist Otto Heinrich Warburg discovered that tumor cells produce more energy than usual and absorb more glucose than healthy cells through glycolysis regulation, a process known as the "*Warburg effect*" (189). Numerous scientific reports support the idea that autophagy can generate ATP through a mechanism that involves glycolysis (**Figure 3**, right panel). Through studies linking these cellular processes, a new paradigm known as the "*reverse Warburg effect*" has emerged. Stromal cells have been postulated as the key generators of fuel for transformed cells (190). Nowadays, both tumor cells and adjacent stromal cells are believed to contribute to meeting tumor energy needs (191). The importance of autophagy in glycolysis has also been observed in chronic myeloid leukemia and breast cancer cells (192, 193). In mice with K-Ras-driven lung tumors, loss of *atg7* leads to defective autophagy, which alters tumor fate, forming benign tumors called oncocytomas (118). These tumor masses with low autophagy levels show defective mitochondria and neutral lipid accumulation (particularly cholesterol esters) due to fatty acid oxidation defects (194).

Mitochondria are central regulators of cell metabolism, which is why they must function correctly. In general, autophagy plays a vital role in the cleaning and quality control of these organelles (195). Both glucose-dependent metabolic pathways and mitochondrial metabolism are essential in tumorigenesis modulation (196). Under hypoxic conditions, pro-apoptotic receptors (BNIP3 and NIX) are activated to induce mitophagy and promote cell survival through HIF-1 regulation (197, 198). In breast cancer cell lines, IGF-I (insulin-like growth factor 1) expression induces BNIP3 expression through a HIF-1-dependent mechanism (199).

Likewise, in glioblastoma cells, PINK1 deletion (the kinase 1 induced by PTEN is a mitochondrial protein of the serine/threonine kinase type) promotes the Warburg effect through ROS and HIF-1 level stabilization. It also reduces PKM2 (pyruvate kinase isoenzyme M2) activity, both regulators of aerobic glycolysis (200). Glycolysis can also be modulated by the interaction between p53 signaling pathways and mitophagy in head and neck squamous carcinoma cell lines (201). Although the molecular mechanism that links these two metabolic regulators has not yet been fully comprehended, Parkin has been found to regulate p53, a ubiquitin ligase that modulates mitochondrial energy metabolism, antioxidant defense, and radiation-induced tumorigenesis (201, 202).

Acetyl-coenzyme A (AcCoA) is a critical metabolic intermediate in autophagy regulation: when its intracellular levels decrease, the autophagic machinery is activated (203). Recent studies in glioblastoma cells have shown that AcCoA increase as a glycolysis product can regulate genes involved in cell migration and adhesion (204). Pancreatic cancers are highly desmoplastic, leading to highly inhospitable environments for cells with high ROS levels, hypoxia, and insufficient nutrient levels (147). Pancreatic stellate cells are a specialized type of fibroblast, commonly found in this type of neoplasm, which contributes to mitochondrial metabolism (205). In the face of stressful conditions, these cells can secrete alanine through an autophagy-dependent mechanism and then be absorbed and used by tumor cells (206). This amino acid fuels the Krebs cycle in PDAC and allows glucose to be used for other anabolic processes such as serine/glycine biosynthesis. Autophagy inhibition in pancreatic stellate cells has recently been shown to decrease tumor growth in transplantation models (149).

HIF-1 and the c-Myc oncogenes coordinated expression regulate cellular glucose transporters, glycolytic enzymes, and mitophagy through choline metabolism (207–209). In B lymphoma cells, c-Myc activates the choline phosphate cytidyltransferase A (PCYT1A) enzyme, inducing mitophagy and preventing cells from dying of necroptosis (210). Arginine is another amino acid that can be dysregulated in cancer cells. Autophagic regulator AMBRA1 (regulator 1 of BECN1 and autophagy) can influence tumor metabolism by regulating c-Myc degradation. When mTOR is inhibited, AMBRA1 is activated. This protein favors the interaction between c-Myc

and its phosphatase PP2A (protein phosphatase 2A) to result in the dephosphorylation and degradation of c-Myc, thus reducing cell division rate (211). Cell transformation mediated by c-Myc or RAS-v12 overexpression increases AMPK and FoxO3 expression, which results in increased levels of positive autophagy for BNIP3 and LC3 (212). Finally, the close link between the signaling mechanisms triggered in tumor cells and the adjacent stroma should be taken into consideration; all these events together contribute to tumor metabolism (206, 213).

AUTOPHAGY DURING VIRAL INFECTION

Autophagy is an essential cellular response element for various types of infections. In general, intracellular pathogens are sequestered and selectively degraded by autophagosomal vesicles (214). However, many pathogens use the host cell's autophagic machinery to survive and spread (68). Viruses are a good example: once inside the cell, they modulate autophagy to regulate almost all viral life cycle, including insertion and entry of the virus into the host cell, exposure of viral components, and viral protein production (215). Some viruses use autophagosomal membranes as anchors in the replication process, while others inhibit autophagy from avoiding being degraded by lysosomal enzymes in autolysosomes (216). A more detailed examination of the molecular mechanisms modulated during viral infection in relation to the autophagy degradation pathway will be made in the following sections.

Autophagy in Antiviral Immunity Regulation

Among the various PRRs involved in detecting pathogenic components, TLR receptors located in the plasma membrane and the cytosol stand out (217) (**Figure 4A**). TLRs activation in endosomes requires PAMPs endocytosis, such as viral RNA, damage-associated molecular patterns (DAMPs), apoptotic cells, or autophagy induction (218). Upon activation, TLRs recruit the Myd88 primary response protein or adapter molecule 1 as an NF-kB activator, contributing to the synthesis of inflammatory cytokines, which in turn trigger IFN production (219) (**Figure 4A**). NF-kB, IRF-3, and IRF-7 activation can determine the inflammasome assembly and ultimately result in caspase-1 and IL-1β, and IL-18 activation (220). On the other hand, astrocytes infected with three different Zika virus (ZIKV) strains show an increase in inflammatory molecule release (IP-10, IFN-β, NF-kB) and autophagy activation by a mechanism involving TLR3 (221).

In mice dendritic cells infected with the human herpes simplex virus-1 (HSV-1), *atg5* deletion causes deficiency in the

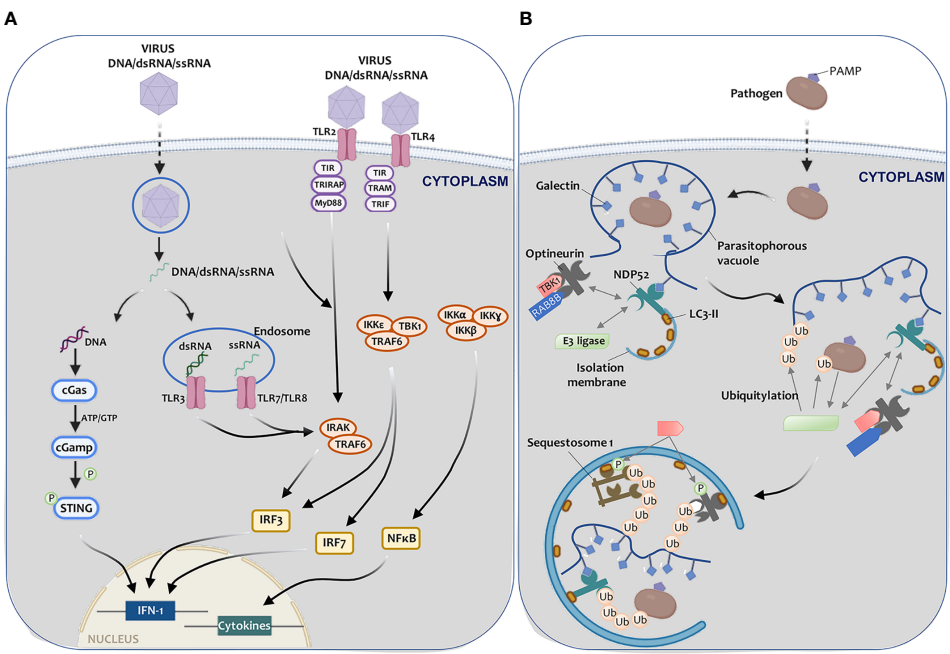


FIGURE 4 | Autophagy-mediated elimination of pathogens. **(A)** Once a virus enters a cell, (I) viral DNA may be exposed in the cytoplasm, leading to cGAS-STING pathway activation and IFN-I expression, thus triggering the antiviral defense; or (II) viral RNA (endosome) can be recognized by TLR3 (dsRNA) and by TLR7 or TLR8 (ssRNA), triggering IRAK and TRAF6, which leads to IFN-I synthesis. Virus recognition by TLR2 or TLR4 may also trigger the cascade that leads to IFN-I (IRF3, IRF7, or NF-kB) and pro-inflammatory cytokine activation transcription factors. **(B)** Once pathogens enter the cell, a parasitophorous vacuole with glycosylated molecules starts enclose them. Galectins present in this vacuole bind to NDP52, which interacts directly with E3 ligase LRSAM1 and indirectly with TBK1. TBK1 interacts with optineurin. NDP52 binds to LC3-II, resulting in xenophagy activation. LRSAM1 polymerize ubiquitin at different targets that are yet to be identified. This hypothetical model includes the pathogen and the parasitophorous vesicle as targets. NDP52 recognizes the ubiquitin tags, optineurin, and sequestosome-I. TBK1 phosphorylates optineurin and sequestosome (I) After these steps, the autophagic isolation membrane elongates to capture the pathogen to degrade it.

processing and presentation capacity of viral antigens into MHC-II. In this process, TLRs located in endosomal membranes and associated with LC3 have been shown to improve viral antigen processing (222). Moreover, *atg5* has been proven necessary for IFN-α production and TLR9 activation through CpG (DNA regions that make up 40% of gene promoters in mammals, with high concentrations of phosphate-linked cytosine and guanine) in HSV-2 infected cells (223).

The second most important PRRs family are the NOD receptors (224), which constitute the cytosolic counterpart of TLRs and include 23 members in humans (225). NOD1 and NOD2 are two receptors that recognize peptidoglycan, a component of the bacterial cell wall; the stimulation of these two receptors forms a transducer complex called the NOD signalosome (226). This signalosome leads to NF-kB activation, which stimulates chemokines and cytokines production, and which in turn initiates the pro-inflammatory response involved in eliminating pathogens (227). Recent studies have revealed an increase in NOD1 and ATG5-dependent autophagy in hepatic ischemia/reperfusion injuries (228). On the other hand, epithelial cell infection with adherent/invasive *Escherichia Coli* have increased ATG16L1 and LC3 through interaction with NOD2 cytosolic receptor (229).

The CRL receptors family binds to carbohydrates present in pathogens (lectins are proteins that recognize sugars) through a Ca^{2+} dependent mechanism (230). Type C lectin receptor, Mincle (macrophage-inducible Ca^{2+} dependent lectin receptor), and TLR4, induce autophagy by activating Myd88 in macrophages (231). RIG-I receptors are characterized by having a C-terminal regulatory domain and a DExD/H helicase domain that contribute to recognizing and unwinding the viral RNA duplex (232). Melanoma differentiation-associated protein 5 (MDA5) is another essential protein in this family. Both proteins detect viral RNA in the cytosol by interacting with mitochondrial signaling through its caspase recruitment domain and regulating autophagy (233).

The last group of receptors includes cytosolic DNA and RNA sensors. An excellent example of these sensors is the cyclic GMP-AMP synthase (cGAS), which plays a fundamental role in recognizing DNA viruses and IFN pathway induction (234). Excessive IFN stimulation can damage the body, so complex regulatory mechanisms have been developed, one of them being autophagy (235). After infection with HSV-1, BECN1 interacts with cGAS, thus altering its nucleotidyltransferase function and triggering the autophagic machinery. When free in the cytosol, cGAS recognizes virus DNA and activates IRF-3 and STING (interferon gene stimulator) to increase IFN production (236) (**Figure 4A**).

Autophagy can also modulate the adaptive immune response to infection through intracellular pathogens. MHC-I molecules are responsible for presenting pathogenic antigens to CD8+ T lymphocytes in order to trigger different cellular responses, such as endocytosis, vesicle trafficking, and autophagy (237). In the conventional mechanism, the proteasome breaks down these protein antigens into peptide fragments and transports them to the endoplasmic reticulum, where their processing concludes (238) (**Figure 4B**). Some cells, such as dendritic cells, can present pathogenic antigens to lymphocytes through "*cross-presentation*" (239). This mechanism can occur through three different signaling pathways (240). In the first case, pathogens are recognized and transported to the proteasome, where small peptide fragments are released and transported to the ER by TAP1 (transporter associated with antigen processing 1) and TAP2 to be presented to HMC-I molecules (240) (**Figure 4B**). The second pathway is independent of the proteasome, and its lysosomal proteolysis helps facilitate antigen processing (241). Finally, in the last signaling pathway, degradation is proteasomal but independent of TAP (242).

The various death mechanisms involved in dendritic cell cross-presentation have been assessed in comparative studies, which show that *atg5* inhibition also inhibited said presentation. This finding has made it possible to associate autophagy with antigens effective presentation to CD8 + T cells (243). Subsequent studies have revealed that autophagy not only influences antigen processing on HMC-I molecules but that is also a prime antigen source for HMC-II molecules, such as CD4 + T cells (244).

Viruses Can Activate or Inhibit Autophagy in Favor of Their Replication

Viruses are particles that cannot survive on their own. This is why they have evolved alongside their respective hosts, a process that has given them the ability to use host cell signaling pathways to their advantage (245). To this end, viral particles promote the expression of various viral proteins that mimic host protein structure and function (215). These proteins modulate many cell signaling pathways in favor of viral replication, and autophagy is not exempt from this regulation (246, 247). Viruses with RNA in their genetic material usually contribute to autophagic membrane accumulation, regardless of whether their replication is nuclear or cytosolic (248).

Many viruses that regulate autophagy to facilitate viral survival and replication have been discovered, including poliovirus (249, 250), Coxsackievirus (CVB3) (251, 252), CVB4 (253), Enterovirus 71 (EV71) (254), human rhinovirus (HRV) (255), foot-and-mouth disease virus (FADV) (256), encephalomyocarditis virus (EMCV) (257), dengue virus (DENV) (258, 259), ZIKV (260, 261), mouse hepatitis virus (MHV) (262), Newcastle disease virus (NDV) (263), severe acute respiratory syndrome coronavirus (SARS-CoV) (264), Chikungunya virus (CHIKV) (265), and Japanese encephalitis virus (JEV) (266), among others.

In cells infected with human poliovirus, viral proteins 2BC and 3A promote the formation of autophagic vesicles where viral replication takes place. The virus induces tubular structures in early stages of infection, while forming double-membrane vesicles in advanced settings (267, 268). Recent studies have provided a novel and deeper understanding, by revealing that these viruses can regulate autophagy through a ULK1-independent mechanism (269). During infection with EV71, the ERK inhibition pathway and autophagy impairs viral replication (270).

Measles virus (MeV) belongs to the *Paramyxoviridae* family and manifests itself mainly in children as high fever, acute respiratory infections and typical papular rashes (271). MeV binds to the host cell through CD46 (CD46 complement regulatory protein), a receptor on the plasma membrane that initiates the autophagic cascade when activated (272). Once active, this receptor binds to the VPS34-BECN1 complex *via* the GOPC scaffold protein (containing Golgi-associated PDZ and spiral-spiral motif) (273). Virulent MeV samples recognized by CD150 membrane receptors have not shown to have the ability to regulate autophagy in early stages of infection. However, these strains modulate autophagy late in the mechanism to prevent cell death and benefit viral replication (274).

The human immunodeficiency virus (HIV) is a member of the *Retroviridae* family, affecting more than 30 million people worldwide (275). This human disease progresses towards immune system failure, resulting in infection development or tumor transformation (276), generating alterations in the host's signaling pathways, and therefore damage accumulation. Critical regulation aspects of the cell cycle are altered, which determine cell transformation and tumor progression, mainly of B cells (277). HIV tat protein, HIV-induced immunosuppression, and a hyperinflammatory state facilitate the oncogenic activity of Kaposi's sarcoma-associated oncovirus (278). Average survival after HIV infection is estimated to be nine to eleven years without treatment, depending on HIV subtype (279). This retrovirus is transmitted by body fluids and infects CD4+ lymphocytes and macrophages, ensuring prevalence in the host through complex cellular processes (280). In macrophages, autophagy can contribute to HIV degradation or replication. Nef viral protein blocks autophagy initiation by promoting BECN1 binding to Bcl-2 through a PRKN-ligase dependent mechanism (Parkin RBR E3 Ubiquitin Protein Ligase) (281). In studies on CD8+ lymphocytes infected with HIV from patients with lymphoblastic leukemia, the virus was able to inhibit autophagy by reducing ATG8 and BECN1. The opposite result was seen in HIV-infected CD4+ cells (282). Furthermore, an active modulation of ATG1, ATG4D, and ATG5-ATG12 proteins by the virus has been shown. The findings reported so far indicate that HIV can modulate autophagy at different stages to benefit its replication and escape cell degradation (215, 283).

The group of RNA viruses belonging to the *Coronaviridae* (CoV) family has recently gained relevance. SARS-CoV-2 is the etiological agent of COVID-19, a disease that has had a devastating impact in the past year around the world (284). Before COVID-19, six human CoV pathogens had been identified, two of which are aggressive enough to develop massive infections (285). SARS-CoV infected cells can use the autophagic machinery to degrade viral particles or promote their replication and prevalence. However, more studies are needed to better understand the signaling pathways involved in these viral replication processes (286).

The influenza virus (family *Orthomyxoviridae*) is another group of viruses implicated in developing human respiratory diseases. Three influenza serotypes have been characterized, only one of them responsible for generating epidemics annually and pandemics at irregular intervals (287). Viral protein M2 is a proton channel that facilitates the acidification of viral particles and allows their decomposition in the host cell nucleus (288). This protein also blocks autophagosome degradation and redirects LC3 to the plasma membrane, generating a cellular redistribution of membranes coupled with this protein. Through this process, these viruses can lead to the formation of filamentous buds, which appear to increase virus stability (289).

CHIKV is transmitted to humans by the bite of some mosquito species and can induce the autophagic machinery through ER stress, increased ROS levels, and reactive nitrogen species (290). Some studies have reported that when the virus is actively replicating, it induces autophagy through the AMPK pathway. However, this has not been observed when the virus is latent (291). Another virus transmitted to humans by mosquitoes is DENV, a *Flaviviridae* family member that can cause acute or chronic infections (292). These viruses replicate in ER invaginations, so autophagy does not have a structural role in replication. However, cells activate lipophagy to break down cellular triglycerides as well as increase B oxidation and energy production (293). Notably, infections caused by ZIKV, an RNA flavivirus, has generated epidemic outbreaks throughout the world from 2007 to the present (294). A recent study has shown that autophagy can facilitate viral replication through autophagosomal vesicle production or inhibit it in *in vivo* and *in vitro* models (295)

Oncovirus and Autophagy

Traditionally, cell transformation has been associated with chronic exposure to various carcinogens, such as ionizing radiation and chemical carcinogens, or to genetic predisposition. However, scientific evidence linking viral infections to tumor development has increased (296). Currently, between 15% and 20% of neoplasms are considered to be related to primary viral infections (297). These oncogenic viruses integrate into the host cell genome and utilize host signaling pathways to regulate cell proliferation and differentiation, genomic stability, apoptosis, and immune system recognition (298, 299). Oncoviruses can be classified as direct and indirect carcinogens, although there is some overlap between both. Indirect regulation is related to chronic inflammation modulation contributing to carcinogenesis (300).

Human viral oncogenesis is a complex process in which only a low percentage of individuals develop cancer years after viral infections (301). During this coexistence between the virus and the host, multiple genetic and epigenetic alterations accumulate, contributing to oncogenic pathway dysregulation. In this context, oncoviruses are considered necessary but not sufficient to determine cell transformation (302). Patients with this type of cancer have reduced viral replication, which is required for the cell to actively divide. The virus remains within the cell as a naked nucleic acid in the form of a plasmid or an episome, or it integrates into the cell genome and remains latent (303). Co-evolution of viruses with hosts has shown that the autophagic machinery can be used alongside various proviral and antiviral functions, depending on virus type, cell, and cellular environment (304, 305).

Up to date, eight different oncogenic viruses have been characterized (**Table 1**) and are described as follows. The human papillomavirus (HPV) and the Merkel cell polyomavirus (MCPyV) are both involved in the development of neoplasms associated with

TABLE 1 | Oncogenic viruses and their involvement in autophagy.

Virus	Genetic Material	Capsid	Involvement in autophagy
HHV-8 (KSHV)	dsDNA	Icosahedral capsid embedded in integument, surrounded by a lipid envelope with glycoproteins	Latency: autophagy is decreased - vFLIP inhibits activation of ATG3 and LC3 proteins - LANA binds to viral and host DNA ensuring its stability and inhibiting autophagy Lytic cycle: autophagy is activated - mTORC1 is required for RTA synthesis and viral transport
HHV-4 (EBV)			Latency: PI3K/Akt stimulation inhibits autophagy - LMP-1 activates mTOR to suppress autophagy and facilitate cell growth and proliferation Lytic cycle: autophagy is activated - BALF0 and BALF1 are expressed, both with a region of interaction with LC3 necessary to modulate autophagy induction - RTA promotes autophagy through regulation of ERK1/2
HTLV-1	ssRNA		Early stages: autophagosomes accumulation that contributes to viral replication - Tax protein stimulates BECN1 and NFkB leading to formation of autophagosomes - p47 degradation induces NFkB Late stages: autophagy inhibition - BHZ activates mTOR, blocking autophagosomes binding to lysosomes
HCMV	dsDNA		Primary infection: autophagy is induced - Increased levels of LC3 - TLR2 activation by glycoproteins in the viral envelope One day after primary infection: - TRS1 and IRS1 associate with BECN1 inhibiting autophagy
HCV	ssRNA	Lipid bilayer with 2 glycoproteins: E1 and E2. A non-icosahedral nucleocapsid surrounds the lipid bilayer.	It has been reported an increase in the number of autophagosomes accompanied by decrease of their degradation. Autophagic machinery is necessary for viral translation but becomes expendable once the viral replication process begins. Selective autophagy is induced in some organelles such as mitochondria and lipid deposits in cells. ATG5-12/16L1 protein complex is recruited to membrane, contributing to viral replication.
HBV	dsDNA		SHB protein generates a massive induction of autophagy HBx induce autophagosome formation through BECN1 modulation HBx represses V-ATPase, decreasing acidification of lysosomes, inhibiting autolysosome degradation, contributing to viral development.
HPV	dsDNA		Primary infection can induce autophagic machinery (ATG7, BECN1, and p62 are induced). After primary infection, mTOR is activated, suppressing autophagy, what protects capsid degradation in autophagosomes

mucosa and skin (306). Hepatitis B virus (HBV) and hepatitis C virus (HVC) are associated with 80% of hepatocellular carcinomas (HCC) (307). The herpes virus associated with the development of Kaposi's sarcoma (KSHV) and the Epstein-Barr virus (EBV) are mainly viruses associated with endothelial carcinogenesis (308). Cytomegalovirus (HCVM) is another herpes virus that has an onco-modulatory function (309). And human T-cell lymphotropic virus-1 (HTLV-1) is an RNA retrovirus responsible for the development of adult T-cell tumors (ATLL) (310). These viruses can modulate oncogenic responses by regulating autophagy at different points (300, 311) (**Figure 5**).

HUMAN HERPESVIRUS

Herpesviruses are biological structures that have linear double-stranded DNA, ranging from 100 to 200 kb. The viral genome is packaged in an icosahedral capsule embedded in a protein integument and surrounded by a host-derived lipid envelope (312). The viral envelope has glycoproteins that mediate the union with the cytoplasmic membrane of the host for subsequent entry of the virus accompanied by the nucleocapsid and the integument. The capsid travels through microtubules until it reaches the nucleus, coupled with protein complexes that allow nuclear pore opening. It then injects its genetic material into the nucleus (313).

Eight herpesviruses have been identified in humans with a common evolutionary origin, classified according to their genomic sequence and biological characteristics into three subfamilies (alpha, beta, and gamma) (314). Within the gamma-herpesviruses, we can find human herpesviruses 4 (EBV) and 8 (KSHV). These viruses have a high affinity for B lymphocytes, and both establish latent and lytic infections that promote the development of hematological diseases and various types of solid human cancers (315). Surprisingly, the ability of viruses to regulate autophagy can vary according to infection stage (316). HCMV is another herpes virus that primarily infects fibroblasts, but which can also be found in epithelial cells, endothelial cells, hepatocytes, stromal cells, monocytes/macrophages, astrocytes, and neural stem/progenitor cells (309).

Kaposi's Sarcoma Virus

KSHV was discovered in 1993 from a tissue sample of a patient with Kaposi's sarcoma (KS) (317). This virus is generally transmitted by body fluids, although transmission has been observed through the placenta to the fetus in some rare

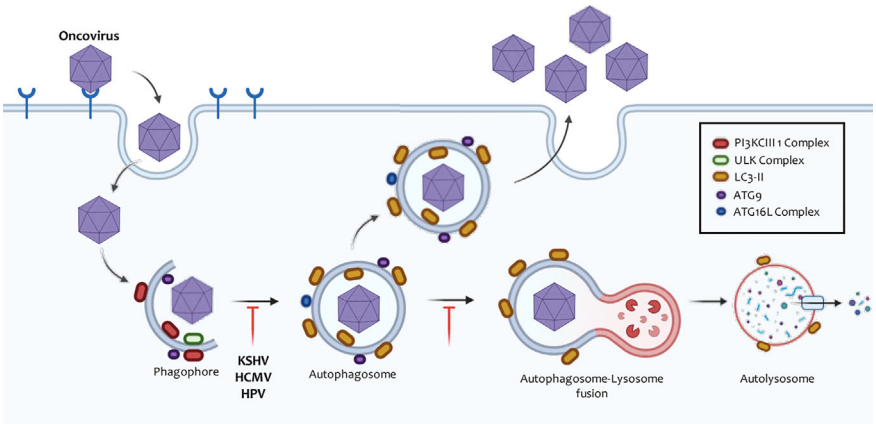


FIGURE 5 | Manipulation of autophagy by oncoviruses. Oncoviruses regulate autophagosome formation or degradation in order to promote their replication cycle. Like many DNA viruses, Herpesviruses prevent their degradation by inhibiting the creation of these vesicles, whereas RNA viruses often induce the accumulation of autophagic membranes independent of their replication in the cytosol or the nucleus. These autophagic membranes can form autolysosomes and finally conclude. In other cases, the autophagic membranes may be used as scaffolds, where the viral replication complexes are positioned or serve as support for the release of the particles. Poliovirus was the first virus where this mechanism has been evidenced.

occasions (318, 319). KSHV seroprevalence is estimated to range between 5%–20% worldwide. Yet, only a small number of patients develop secondary diseases associated with the virus, the population mostly at risk being immunosuppressed individuals or individuals with immune system abnormalities, as is the case of AIDS patients (320). KSHV infection is associated with the development of various human pathologies, including Kaposi's sarcoma, primary effusion lymphoma (PEL), multicentric Castleman's disease (CMD), and inflammatory cytokine syndromes (KICS) (321). The main reservoir of this virus in its latent form are B lymphocytes, but it can also infect monocytes, fibroblasts, endothelial, epithelial, and dendritic cells through association with receptors on the plasma membrane (322).

After primary infection, the virus remains in the cell as an episome and regulates various host signaling pathways to replicate correctly. The virus produces proteins associated with the viral latency phase which are essential for cell transformation (323). Some of the encoded proteins are complement-fixing proteins (v-CBP), viral interleukin-6 (v-IL-6), viral inflammatory protein type-I (v-MIPI) and type-II (v-MIPII), viral Bcl-2 (v-Bcl-2), viral interferon regulatory factor (v-IRF), viral cyclin (v-Cyclin), latency nuclear antigen (LANA), viral adhesin (v-ADH), receptor-coupled G-protein (v-GCR), thymidylate synthetase, thymidine kinase, ribonucleotide reductase (300, 324)

In various models, autophagy has been found to be a cellular mechanism commonly regulated by viral KSHV proteins (325). After initial expansion at the infection site, the new viral particles spread throughout the body and reach the cells in order to establish a latency phase, especially in the B lymphocytes and endothelial cells of the blood/lymphatic vessels (326). During the latency phase, autophagy and other cellular mechanisms contribute to creating a cellular microenvironment favorable to tumor initiation and progression (321).

The transmembrane glycoprotein K1, encoded by the first KSHV open reading frame, is a signaling protein capable of causing B cell activation (327). V-cyclin and K1 have been found to promote autophagy by stimulating the AMPK pathway (328, 329) (**Figure 6**). On the other hand, vFLIP restricts the autophagic machinery by inhibiting ATG3 and LC3 proteins (330) (**Figure 6**). Once viral latency is established, LANA plays a fundamental role in maintaining this phase through NF-kB activation (331). Granato et al. observed viral particles inside autophagic vesicles in the cytoplasm of PEL cells in active replication, thus postulating that autophagy may also be related to viral transport (332). Finally, another protein linked to viral latency is STAT3 (signal transducer and activator of transcription 3), which remains active in a state of viral latency (330). In dendritic cells, KSHV infection induces STAT3 phosphorylation, promoting cell survival and viral latency. Moreover, the release of IL-10, IL-6, and IL-23, cytokines that contribute to keeping STAT3 active, is also induced (333). This allows the viral genome to remain unchanged and the particles to replicate successfully (334).

Numerous cellular events can activate lytic KSHV reactivation. Cellular microenvironment can shape the viral epigenome to facilitate latency reactivation (335). ER stress can induce *rad21* cleavage, a member of the cohesin complex that generates dramatic changes in the KSHV genome. The loss of DNA loops triggers virus reactivation (336). During this process, cells activate the autophagy machinery with various functionalities (335).

A fundamental component for lytic reactivation is the expression of lytic switch master proteins (RTA), targeting 100 different sites in the KSHV genome and transactivating 34 lytic

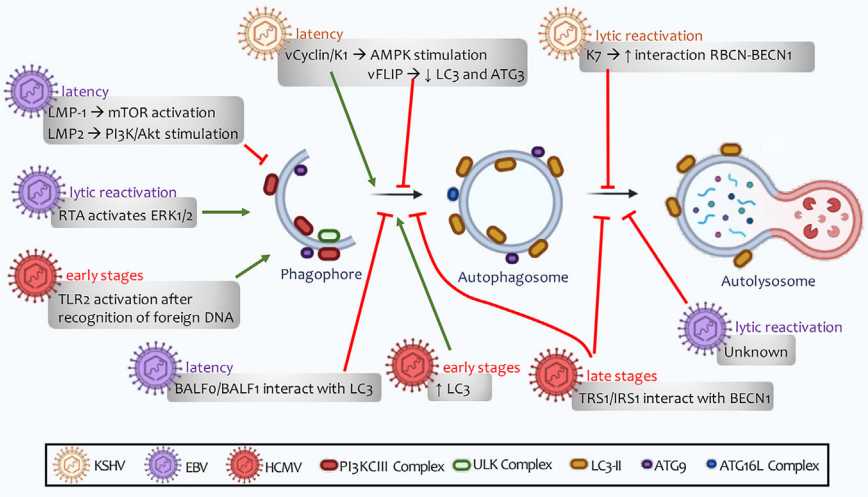


FIGURE 6 | Human Herpesvirus regulation of autophagy. The viral ability to regulate autophagy depends on stage of infection. This regulation can alternate between latent, *de novo*, or lytic reactivation infections. A distinguishing feature of these lymphotropic viruses is their ability to promote autophagy induction, a process followed by inhibition of autophagosome maturation. This defective autophagy does not allow autolysosomes to form, and therefore to perform successful clearance.

genes (337). Pringle et al. have reported that mTORC1 is required for lytic replication and RTA synthesis as an activator of cellular transcription. In contrast, this autophagy inhibitor complex has not shown more significant involvement in genomic replication, late gene expression, or in the release of infectious progeny (338). Furthermore, autophagy inhibition has been reported to reduce lytic KSHV reactivation (339).

In contrast to the nuclear LANA function, cytoplasmic isoforms of this viral protein mediate lytic reactivation by antagonizing cellular DNA sensors. These isoforms bind to cGAS, a process that involves STING and NF-kB induction (340). Viral protein K7 expression stimulates RBCN (Rubicon autophagy regulator) interaction with BECN1. These events promote the blocking of autophagosome maturation (333) (**Figure 6**). KSHV monocyte infection counteract ROS increase induced by macrophage colony-stimulating factor (M-CSF), preventing JNK and Bcl-2's phosphorylation and inhibiting autophagy. Together with the decrease in TNFα and the increase in the immunosuppressive cytokine IL-10, all these events lead to impaired macrophage survival and differentiation (341). Findings so far allow us to infer that these viruses may induce autophagosomes formation, in which they are transported to the cell surface. Viral proteins inhibit lysosome-to autophagosome-binding and, therefore, autolysosome formation is not degraded by lysosomal hydrolases (330) (**Figure 6**).

Epstein Barr-*Virus*

EBV is a very easily transmitted herpesvirus that is mainly contracted in childhood through body fluids. 90% of the world's population is believed to have been infected at some point of their lives (342, 343). This virus has contributed to the development of various secondary pathologies, such as infectious mononucleosis and some neoplasms of epithelial and lymphocytic origin (344). After entering host cells, viruses amplify and rapidly enter a state of latency. A distinct fact is the presence of three different latency types that can be independently regulated, autophagy playing an essential role in this regulation (345, 346). Each cell presents multiple copies of viral DNA episomes and produces a series of proteins associated with latency, including six nuclear antigens (EBNA 1, 2, 3A, 3B, 3C, and -LP) and three latent membrane proteins (LMP1, 2A and 2B) (347).

LMP2 viral protein has been reported in most neoplasms associated with this virus. This protein stimulates the PI3K/Akt signaling pathway, a process that triggers apoptosis and autophagy inhibition, thus contributing to tumor cell proliferation (348) (**Figure 6**). This regulation has been seen in gastric carcinomas associated with EBV infection, where the presence of PI3K mutations has been associated with higher tumor occurrence and metastasis (349). Furthermore, LMP2 has been found to contribute to cell proliferation through p27 degradation (350, 351).

Additionally, LMP1 binds to membrane-bound death receptors TRAF and TRADD (tumor necrosis factor receptor type 1-associated death) or activate signaling pathways that include NF-kB, JNK, p38, small GTPases (Cdc42), and the JAK/AP-1/STAT cascades. Moreover, LMP-1 has been reported to activate mTOR to suppress autophagy and facilitate tumor growth and proliferation (352) (**Figure 6**). In contrast, Hurwitz has shown that LMP-1 can inhibit mTOR by secreting CD63-dependent vesicular proteins, contributing to autophagy induction. This induction is not complete since LMP1 inhibits lysosomes' binding to autophagic vesicles in order to avoid the viral particles' degradation by lysosomal hydrolases (353). On the other hand, EBNA3C nuclear antigen activates autophagosome formation through transcriptional induction of several autophagy regulators, including ATG3, ATG5, and ATG7 (354).

During the EBV lytic cycle, autophagy may present bimodal modulation, showing an early stimulation phase in combination with the inhibition of the late phases of the autophagic mechanism (degradation of cytoplasmic material by lysosomal hydrolases) (355). This final regulation favors the acquisition of envelopes and components of the autophagic machinery by newly synthesized virions (356, 357). The viral proteins associated with this virus that regulate autophagy in the EBV lytic cycle have not yet been fully characterized. RTA function, an early expression protein regulating autophagy through an ERK-dependent mechanism, has been recently highlighted (358) (**Figure 7**).

EBV codes for the expression of two Bcl-2 homologous viral proteins called BHRF1 and BALF0/1 (359, 360). Both viral proteins prevent apoptosis during early B-cell infection but may be dispensable once the latent infection is established (361). BHRF1 anti-apoptotic activity has been studied extensively (362). However, BALF0/1 expression and function remain ambiguous. Two in-frame methionine codons are present near the beginning of the BALF0/1 open reading frame (ORF), suggesting that two proteins with different N-termini may be encoded (363). So far, the BALF1 protein is known to be encoded by the shortest ORF, while the BALFO protein is encoded by the first non-conserved methionine. During the lytic cycle initial phase, BALF0 and BALF1 are expressed, both with a region of interaction with LC3, and modulate autophagy induction (364). So far, we can infer that EBV inhibits autophagy during the latency phase in its initial steps. Simultaneously, during lytic reactivation, the formation of dysfunctional autophagosomes is promoted (**Figure 6**).

Human Cytomegalovirus

HCMV is a double-stranded DNA herpes virus transmitted through body fluids, not prevalent in any particular age range. This virus has an extensive genome of 236 kilobases, one of the largest viruses that can infect humans (365). Primary infections are generally asymptomatic, although congenital diseases can lead to various severe disabilities or fetal death (309).

HCMV particles have been detected in different cell types, including epithelial cells, connective tissue, hepatocytes, various populations of leukocytes, and vascular endothelial cells (366). HCMV also infects tumor cells and contributes to transformation when affecting healthy tissue by modulating various cellular signaling pathways (309). This virus regulates autophagy in a bimodal manner (367). First, during early stages of infection, it induces autophagic vesicle formation. Later in infection, HMCV blocks autophagy through viral proteins synthesized in the host cell (368). Two proteins involved in autophagy inhibition by association with BECN1 (TRS1 and TRS2) have been identified (**Figure 6**). Mouna et al. has found that co-expression of viral proteins TRS1 and IRS1 is essential for autophagy inhibition in various cell models (369).

Recent attention has been given to viral components that determine HMCV latency and lytic reactivation, with special focus on the *uLb'* gene locus (*ul133-138*) that restrict viral replication by modulating viral latency and immune evasion through the expression of a considerable number of viral proteins (370). An example of this is UL138: this viral protein is presented to HMC-I to regulate the host's adaptive immunity in fibroblast, and the autophagic machinery holds this event (371). In contrast, it was reported that autophagy inhibition generates a high response of CD8 + lymphocytes due to the internalization of molecules in MHC-I (372).

Early expression of viral proteins associated with HMCV genes 1 and 2 (IE1 and IE2) is necessary for lytic reactivation of host cell virus and immunomodulation (373). IE2 can interact with itself and UL84 as well as with many specific cell

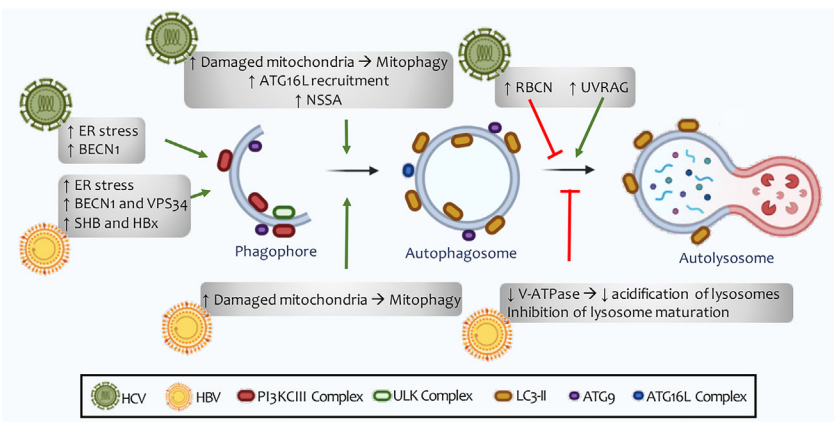


FIGURE 7 | HCV and HBV regulation of autophagy. The autophagic machinery is necessary for incoming viral RNA translation, but it becomes expendable once the viral replication process begins. An increase in the number of autophagosomes does not correlate with an increase in the rate of lysosomal degradation. HCV has been shown to induce the expression of Rubicon, thus inhibiting the maturation of autophagosomes. For its part, HBC reduces the acidification of lysosomes and autolysosomes through the inhibition of V-ATPase (H + type vacuolar enzyme). However, *in vitro* studies show good fusion of autophagosomes with lysosomes, as well as successful autophagic flow, indicating that these viruses are able to induce selective autophagy in some cellular organelles, such as the mitochondria and lipid deposits in cells.

transcription factors to regulate gene expression. This protein plays a critical role in viral DNA synthesis and is also considered to counteract host response (374, 375). IE2 overexpression has been recently found to induce autophagy in HMCV-infected cells (376). These results show that in the early stages of HCMV infection, viral proteins contribute to autophagosomal vesicle formation. At the same time, they inhibit vesicles-to-lysosomes binding in later stages, thereby losing their degradative capacity (**Figure 6**).

HUMAN T-CELL LYMPHOTROPIC VIRUS TYPE 1

HTLV-1 is a member of the *Retroviridae* family and is represented by a small single-stranded RNA genome approximately nine kilobases long (377). This retrovirus was first isolated in 1979 in samples from patients with cutaneous manifestations of rapidly growing T-cell lymphoma (ATLL) (378). Furthermore, HTLV-1 is associated with the development of poliomyelitis, HTLV-1 associated myelopathy, infectious dermatitis, arthropathy, and facial nerve palsy (379). Evidence suggests that there are between 5 and 20 million HTLV-1 carriers worldwide, but only 3-5% of them develop secondary pathologies (380, 381).

Immediately after entering the host cell, the viral RNA undergoes reverse transcription, and then binds to the cell's genome as a provirus. This binding generally occurs in areas close to the binding sites of transcription factors such as STAT1, TP53, and HDAC6 (histone deacetylase 6). Dysregulation generates alterations in cell signaling in the expression of specific genes and autophagy is not exempt from this viral regulation.

Regulatory protein HTLV-1 Tax is an oncoprotein that plays an essential transcriptional role in viral replication and participates in T lymphocytes' transformation. It can also transactivate or transrepression more than 100 cellular genes by linking and modulating stability and activity (300). Recently, HTLV-1 infection has been reported to induce autophagosomes in cells and inhibit their binding to lysosomes through a tax-dependent mechanism. In this way, the number of non-degrading autophagic vesicles, where viruses can replicate, increases considerably (382). Therefore, Tax viral protein, located in the plasma membrane's lipid microdomains, binds to the IKK complex to stimulate BECN1 and NF-kB activity (383).

Cell adhesion molecule 1 (CADM1) is a member of the immunoglobulin superfamily and is considered to be an excellent cell surface marker of HTLV-1 infected T cells (384). For CADM1 to be correctly expressed, Tax and NF-kB induction and p47 (a negative NF-kB regulator) degradation are necessary. Autophagy is p47 primary degradation mechanism, and it is active in most ATLL cells infected with HTLV-1 (385).

Another essential viral protein for ATLL development is HBZ (bZIP factor) (303). HBZ inhibits both apoptosis and autophagy and may induce the expression of brain-derived neurotrophic factor (BDNF) and its receptor (386). According to the subcellular location of HBZ, it contributes to tumor progression (nuclear) or favourably contributes to inflammation induction (cytoplasmic) (387). When HBZ is exported from the nucleus to the cytoplasm, it activates mTOR through the DNA damage-inducible protein PPP1R15A (a regulatory subunit of protein phosphatase -1 15A) (386). As in other viral infections, HTLV-1 induces autophagosome formation and inhibits binding to lysosomes to prevent degradation. Consequently, the number of autophagosomal vesicles in the cytosol increases, creating a physical obstacle for developing other cellular processes and a favorable environment for viral particle formation (388).

HEPATITIS C VIRUS

HCV belongs to the *Flaviviridae* family, characterized by viral particles with an RNA helix of 9.6 kb in length and wrapped in a lipid bilayer with two anchored glycoproteins (E1 and E2). In general, it presents a non-icosahedral nucleocapsid, although it is possible to find viruses without nucleocapsid in infected patients' blood (389). Chronic HCV infection can trigger liver fibrosis and cirrhosis, and it is also associated with hepatocellular carcinoma (HCC) and non-Hodgkin lymphomas development (390). This virus can promote carcinogenesis directly through the modulation of specific signaling pathways and indirectly through chronic inflammation (391).

Once inside the host cell, the virus forms a membranous network used during replication (392). Three membranous rearrangements associated with the virus have been identified: cluster vesicles, contiguous vesicles and double-membrane vesicles. In general, cluster vesicles are associated with viral infection early stages. As the infection progresses, the number of double-membrane vesicles also increases (393).

Some viral proteins (CORE, NS2, NS5B, NS3, NS5A) directly benefit carcinogenesis through the induction of proliferation, angiogenesis, apoptosis, immune response, and inhibition of tumor suppressors (303). HCV can induce autophagosome formation and inhibit binding to lysosomes (311) (**Figure 7**). However, some *in vitro* studies have revealed that the virus can cause successful autophagic flux. This conflicting result may be explained by HCV probably inducing selective autophagy in some cellular organelles, such as the mitochondria and lipid deposits in cells (394).

Furthermore, the ATG16L complex is recruited into the membranous network, contributing to viral particle replication, and subsequent ATG12 removal suppresses viral RNA replication. Autophagy activation can counteract HCV infection, and the virus has developed different strategies to strengthen its persistence by temporarily regulating the autophagic process (395). The autophagic machinery is necessary for the translation of viral RNA in early stages of infection but becomes dispensable later. Once the replication process is complete, autophagy contributes to releasing viruses to the extracellular space, thus benefiting HCV transmission (300).

HCV infection has been found to induce autophagy through the direct interaction of viral proteins with autophagy effectors. In contrast, HCV has been shown to induce stress autophagy of the endoplasmic reticulum by inducing the three response pathways to misfolded proteins (p-ERK, ATF6 (activating transcription factor

6), IRE1) (396) (**Figure 7**). Reticulum stress produces calcium release, which disrupts mitochondrial activity and leads to ROS accumulation and damaged mitochondria. This leads to mitophagy activation through NS5A expression. This HCV non-structural protein causes an increase in LC3 levels and reduces p62 in host cells (397) (**Figure 7**). In patients with chronic HCV infections, mitophagy was observed to be induced due to PINK1 and Parkin's translocation to the mitochondria outer surface. This mechanism correlates with oxidative phosphorylation dysregulation and mitochondria depletion, contributing to liver injury (398). HCV modulates the autophagic machinery in order to exist in the host cell. Like other viruses, it increases the formation of dysfunctional autophagic vesicles within which it replicates. Furthermore, in tumors associated with this virus chronic infections, high levels of mitophagy were presented (**Figure 7**).

HEPATITIS B VIRUS

HBV belongs to the *Hepdnaviridae* family and is responsible for developing acute and chronic viral hepatitis as well as long-term complications ranging from fibrosis and cirrhosis to cancer. Chronic infection progression is predominant in infected patients during the perinatal and infantile periods (399). Two billion people are estimated to be infected with HBV worldwide, and more than 350 million to be chronic carriers. Only 25% of infected patients develop liver neoplasms (300).

These viruses present circular double-stranded DNA of 3.2 kb in length, which has four open reading frames and has the peculiarity of forming an incomplete chain (400). The end of one strand is associated with viral DNA polymerase (401). Immediately after entering the cell, the virus undergoes reverse transcription within the nucleocapsid. Consequently, linear DNA is formed and secreted as virions or transported to the nucleus, integrating into the host genome to regulate tumor transformation (402).

HBV has a complex replication cycle and needs to encode at least seven viral proteins that regulate different processes in the host cell in order to complete it successfully (403). HBx (hepatitis B virus protein x) and SHB (small surface protein) expression is associated with autophagy regulation in several biological models (404) (**Figure 7**). SHB protein travels through the cytosol to the endoplasmic reticulum, where stress increases and generates massive autophagy induction. Signaling pathways initiated by stress sensors such as IRE1, p-ERK, and ATF6 help regulate cell homeostasis (405).

As for HBx, it is a multifunctional regulatory protein that may be involved in viral pathogenesis and carcinogenesis (406). Molecular studies have revealed that HBx generates strong autophagy induction by activating DAPK (death-associated protein kinase) and increasing BECN1 (407). However, in later stages of the autophagic mechanism, HBx can repress V-ATPase (vacuolar enzyme type H+), thus reducing lysosome and autolysosome acidification (408) (**Figure 7**). As they become less acidic, autolysosomes lose their degradative capacity as cytoplasmic debris increases in the cell, creating an inhospitable environment that contributes to carcinogenesis (408). The virus can also interfere with autophagic degradation through RAB7 (Ras-related protein), a small GTPase involved in autophagosome maturation and their fusion with lysosomes (409).

HBV-infected patient biopsies showed that viral persistence is correlated with the expression of mitophagy effectors, Parkin, and PINK (410). Furthermore, this mechanism is believed to be regulated by the AMPK/mTOR/ULK1 axis (411). Liver cells infected with HBV show that miR-155 expression contributes to viral replication and enhances autophagy induction (412). At least four miRNAs have been identified to inhibit viral replication in clinical samples from virus-infected patients, (let-7, miR-433, miR-345, miR-511) (413).

Liver cancer is a global concern due to its high resistance to chemotherapeutic drugs, which has been linked to exosome formation in transformed cells (414). These structures increase drug resistance by inducing chaperone-related signaling pathways and LAMP-2-dependent (type-2 lysosomal membrane protein) autophagy. Patients with liver tumors associated with primary HBV infection show greater tumor volume and greater pathogenicity. This suggests that the virus contributes to generating a more aggressive and resistant HCC phenotype (415).

Therefore, we can infer that HBV generates bimodal autophagy regulation: first by inducing the mechanism and then by inhibiting the formation of mature autolysosomes, thus contributing to viral replication and carcinogenesis (**Figure 7**).

PAPILLOMAVIRUS AND HUMAN POLYOMAVIRUS

Papillomaviruses (HPV) and human polyomaviruses (PyV) were initially considered members of the same virus family due to their morphological similarity and genome organization, but have now been classified into separate families: *Papillomaviridae* and *Polyomaviridae*, respectively (416), both composed of viruses with a double-stranded circular DNA that encodes various regulatory and structural proteins, some of which have oncogenic properties (417).

Merkel cell polyomavirus (MCPyV) is implicated in developing a very aggressive skin cancer called Merkel carcinoma (418). Most findings about MCPyV oncogenic potential are associated with LT (large antigen T) and sT (small antigen T) expression (419, 420). LT has a J domain (heat shock protein binding domain), a retinoblastoma binding motif (RB, inhibits members of this protein family), and a C-terminus binding to helicase/ATPase domain (required for viral DNA replication) (418). LT's oncogenic role is mainly associated to its high binding affinity to RB, which promotes the sequestration and inactivation of this tumor suppressor (421).

Regarding the sT antigen, it shares a 78 amino acid N-terminal region (includes the J domain) with LT and has a unique C-terminal domain. This antigen is considered to be the main MCPyV-induced oncogenesis regulator (422). LTs unique expression is sufficient to transform fibroblasts *in vitro* (423) and *in vivo* models (424). sT keeps the eukaryotic 4EB-P1 binding protein hyperphosphorylated and inactive, which leads to deregulation of cellular translation

events and contributes to cell proliferation and transformation (422). Through these surface antigen expression, viruses modulate the autophagic machinery and contribute to immunosuppression and viral oncogenesis. In MCC tumors associated with MCPyV infection, sT and LT antigen expression suppresses autophagy through miR-375, miR-30a-3p, and miR30a-5p gene modulation. These microRNAs act on *atg7*, *p62*, and *bcn1* to inhibit autophagic initial stages. These events protect cancer cells from cell death (425).

Human papillomavirus (HPV) is the leading cause of cervical cancer (426). It is also associated with the development of non-melanoma skin cancers, cancer of the head, neck, oropharynx, and the development of various anogenital neoplasms (427). The viral genome is integrated into the host cell's DNA and encodes the expression of early viral genes (E1 to E7) essential for cell replication, transcription, and transformation. These viruses can also regulate the expression of late genes L1 and L2, which encode viral capsid proteins (428).

Binding and internalizing the virus are processes closely related to manipulating the host cell autophagic machinery. The entry of HPV into cells is associated with autophagy suppression through mTOR activation (429). This event promotes mTORC1, 4EB-P1, and S6K1 (ribosomal protein kinase 1) phosphorylation and activation (430) and ULK1 inactivation. This -in turn- contributes to inhibiting the initial steps of the autophagic mechanism (431).

Once internalized in cells, HPV circulates through an endosomal compartment where capsid proteins are degraded within acidified endosomes, and the viral genome enters the nucleus (432). Here, HPV DNA is amplified and maintained as episomes in the epithelium basal cells by mechanisms mediated by E1 and E2 (433). These early expression proteins are involved in various cellular signaling mechanisms. In particular, E5, E6, and E7 oncoproteins modulate the host cell autophagic machinery. Keratinocytes with HPV-16 infection, followed by E5 expression, produce a decrease in LC3 levels and prevent p62 degradation. E5 interferes mechanically with the transcriptional activation of the autophagic machinery, regulating the expression of *bcn1*, *atg5*, *lc3*, *ulk1*, *atg4a*, and *atg7* (434).

Unlike E5, viral gene E6/E7 inhibits autolysosome formation by a mechanism involving p53 (435). HPV-16 and HPV-18 infections are associated with the development of squamous cell carcinomas of the head and neck, two neoplasms with a high incidence worldwide (436). In addition to regulating the autophagic machinery, viral proteins E6 and E7 contribute to p53 and p-Rb degradation of. All these events cause the activation of specific transcription factors that modulate cell fate (437). The information obtained so far reveals that autophagy inhibition promotes HPV life cycle and tumor progression.

CONCLUSIONS

In recent decades, studying the different cellular functions associated with autophagy has kept specialized scientists alert. This degradative mechanism, used by mammalian cells to maintain cell homeostasis, also directly contributes to modulating the progression of various diseases, such as cancer. Cellular context is essential to determine the functionality of autophagy. In general, cells accumulate damage at disease initial stages, affecting critical points in cell cycle regulation and thus determining cell transformation. Cells can therefore activate autophagy to shrink the damaged organelles and counteract the stressful stimuli to which they are exposed and restore normal state. However, in the context of tumor progression and invasion, the situation may be different. Here, cells present multiple alterations in their signaling pathways, which develops an aggressive cell phenotype, active and uncontrolled division, and high metabolism. Tumor cells can then induce autophagy to generate a fuel supply to maintain tumor cell metabolism.

Furthermore, autophagy can regulate tumor angiogenesis and immunity to benefit disease progression. Before infection, cells can eliminate intracellular pathogens by enzymatic digestion in autophagic vesicles. However, many viruses have developed strategies that allow them to bypass host attack and achieve successful replication and permanence. The number of viruses that modulate the autophagic mechanism for their benefit is increasing. In general, these intracellular organisms regulate the autophagic machinery in a bimodal manner. Upon entering cells, they promote autophagosome formation and inhibit binding to lysosomes, thus losing their degradative capacity. Viruses can use these dysfunctional vesicles to replicate within them, while debris and damage accumulate in cell organelles due to the mechanism's inefficiency. Other viruses, on the other hand, directly inhibit the autophagic machinery from preventing its degradation. Dysfunctional vesicle accumulation contributes to cell damage accumulation, which benefits cell transformation and tumor development over time. However, more studies are needed to clarify autophagy's relation to viral infections and tumor development. A thorough understanding of these molecular mechanisms is crucial for developing new antiviral drugs and targeted oncogenic therapies.

AUTHOR CONTRIBUTIONS

AS received an invitation to contribute to the Research topic: New functions of autophagy pathways in cancer. This author designed and wrote the manuscript, MM created the schematic figures and provided critical reading, and OC contributed to the overall design, supervision of the work, and essential reading. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by NIH GRANT 1U54CA221208-01 (to OC), UBACYT GRANT 20020150100200BA (to OC), and PICT GRANT 2015-3436 (to OC). The funders had no role in study design, data collection, and analysis, decision to publish or preparation of the manuscript.

REFERENCES

1. Hegyi G, Vincze G, Szasz A. On the Dynamic Equilibrium in Homeostasis. *Open J Biophys* (2012) 02(03):60–7. doi: 10.4236/ojbiphy.2012.23009
2. Greives TJ, Dochtermann NA, Stewart EC. Estimating heritable genetic contributions to innate immune and endocrine phenotypic correlations: A need to explore repeatability. *Horm Behav* (2017) 88:106–11. doi: 10.1016/j.yhbeh.2016.11.015
3. Bektas A, Schurman SH, Sen R, Ferrucci L. Aging, inflammation and the environment. *Exp Gerontol* (2018) 105(October):10–8. doi: 10.1016/j.exger.2017.12.015
4. Chatterjee N, Walker GC. Mechanisms of DNA damage, repair, and mutagenesis. *Environ Mol Mutagen* (2017) 58(5):235–63. doi: 10.1002/em.22087
5. Costas MA, Rubio MF. Autofagia, una estrategia de supervivencia celular. *Medicina (Buenos Aires)* (2017) 77(4):314–20.
6. Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. *Cell Res* (2014) 24(1):24–41. doi: 10.1038/cr.2013.168
7. White E. The role for autophagy in cancer. *J Clin Invest* (2015) 125(1):42–6. doi: 10.1172/JCI73941
8. Yu L, Chen Y, Tooze SA. Autophagy pathway: Cellular and molecular mechanisms. *Autophagy* (2018) 14(2):207–15. doi: 10.1080/15548627.2017.1378838
9. Anding AL, Baehrecke EH. Cleaning House: Selective Autophagy of Organelles. *Dev Cell* (2017) 41(1):10–22. doi: 10.1016/j.devcel.2017.02.016
10. Noda NN, Inagaki F. Mechanisms of Autophagy. *Annu Rev Biophys* (2015) 44(1):101–22. doi: 10.1146/annurev-biophys-060414-034248
11. Mizushima N. The ATG conjugation systems in autophagy. *Curr Opin Cell Biol* (2020) 63:1–10. doi: 10.1016/j.ceb.2019.12.001
12. Pyo J-O, Nah J, Jung Y-K. Molecules and their functions in autophagy. *Exp Mol Med* (2012) 44(2):73. doi: 10.3858/emm.2012.44.2.029
13. Russell RC, Yuan HX, Guan K-L. Autophagy regulation by nutrient signaling. *Cell Res* (2014) 24(1):42–57. doi: 10.1038/cr.2013.166
14. Ha J, Guan K-L, Kim J. AMPK and autophagy in glucose/glycogen metabolism. *Mol Aspects Med* (2015) 46:46–62. doi: 10.1016/j.mam.2015.08.002
15. Gui L, Liu B, LV G. Hypoxia induces autophagy in cardiomyocytes via a hypoxia-inducible factor 1-dependent mechanism. *Exp Ther Med* (2016) 11(6):2233–9. doi: 10.3892/etm.2016.3190
16. Lai MC, Chang CM, Sun HS. Hypoxia Induces Autophagy through Translational Up-Regulation of Lysosomal Proteins in Human Colon Cancer Cells. Trajkovic V, ed. *PloS One* (2016) 11(4):e0153627. doi: 10.1371/journal.pone.0153627
17. Chen A, Sceneay J, Gödde N, Kinwel T, Ham S, Thompson EW, et al. Intermittent hypoxia induces a metastatic phenotype in breast cancer. *Oncogene* (2018) 37(31):4214–25. doi: 10.1038/s41388-018-0259-3
18. Xia G, Zhu T, Li X, Jin Y, Zhou J, Xiao J. ROS–mediated autophagy through the AMPK signaling pathway protects INS–1 cells from human islet amyloid polypeptide–induced cytotoxicity. *Mol Med Rep* (2018) 18(3):2744–52. doi: 10.3892/mmr.2018.9248
19. Han X, Zhong Z, Kou J, Zheng Y, Liu Z, Jiang Y, et al. ROS Generated by Upconversion Nanoparticle-Mediated Photodynamic Therapy Induces Autophagy Via PI3K/AKT/ mTOR Signaling Pathway in M1 Peritoneal Macrophage. *Cell Physiol Biochem* (2018) 48(4):1616–27. doi: 10.1159/000492283
20. Yorimitsu T, Klionsky DJ. Endoplasmic Reticulum Stress: A New Pathway to Induce Autophagy. *Autophagy* (2007) 3(2):160–2. doi: 10.4161/auto.3653
21. Nie T, Yang S, Ma H, Zhang L, Lu F, Tao K, et al. Regulation of ER stress-induced autophagy by GSK3β-TIP60-ULK1 pathway. *Cell Death Dis* (2016) 7(12):e2563–3. doi: 10.1038/cddis.2016.423
22. Corona Velazquez AF, Jackson WT. So Many Roads: the Multifaceted Regulation of Autophagy Induction. *Mol Cell Biol* (2018) 38(21):1–14. doi: 10.1128/MCB.00303-18
23. Jang M, Park R, Kim H, Namkoong S, Jo D, Huh YH, et al. AMPK contributes to autophagosome maturation and lysosomal fusion. *Sci Rep* (2018) 8(1):12637. doi: 10.1038/s41598-018-30977-7
24. Jung CH, Ro SH, Cao J, Otto NM, Kim DH. mTOR regulation of autophagy. *FEBS Lett* (2010) 584(7):1287–95. doi: 10.1016/j.febslet.2010.01.017
25. Shao X, Lai D, Zhang L, Xu H. Induction of Autophagy and Apoptosis via PI3K/AKT/TOR Pathways by Azadirachtin A in Spodoptera litura Cells. *Sci Rep* (2016) 6(December 2015):1–12. doi: 10.1038/srep35482
26. Bourouis M, Mondin M, Dussert A, Leopold P. Control of basal autophagy rate by vacuolar peduncle. Trajkovic V, ed. *PloS One* (2019) 14(2):e0209759. doi: 10.1371/journal.pone.0209759
27. Rabanal-Ruiz Y, Otten EG, Krolchuk VI. mTORC1 as the main Gateway to autophagy. *Essays Biochem* (2017) 61:565–84. doi: 10.1042/EBC20170027
28. Cork GK, Thompson J, Slawson C. Real Talk; The Inter-play Between the mTOR, AMPK, and Hexosamine Biosynthetic Pathways in Cell Signaling. *Front Endocrinol* (2018) 522(9):1–9. doi: 10.1111/odi.12254
29. Klionsky DJ, Schulman BA. Dynamic regulation of macroautophagy by distinctive ubiquitin-like proteins. *Nat Struct Mol Biol* (2014) 21(4):336–45. doi: 10.1038/nsmb.2787
30. Zaza G, Granata S, Caletti C, Signorini L, Stallone G, Lupo A. mTOR Inhibition Role in Cellular Mechanisms. *Transplantation* (2018) 102(>2S Suppl 1):S3–S16. doi: 10.1097/TP.0000000000001806
31. Li X, He S, Ma B. Autophagy and autophagy-related proteins in cancer. *Mol Cancer* (2020) 19(1):12. doi: 10.1186/s12943-020-1138-4
32. Heras-Sandoval D, Pérez-Rojas JM, Pedraza-Chaverri J. Novel compounds for the modulation of mTOR and autophagy to treat neurodegenerative diseases. *Cell Signal* (2020) 65:109442. doi: 10.1016/j.cellsig.2019.109442
33. Qin L, Wang Z, Tao L, Wang Y. ER stress negatively regulates AKT/TSC/mTOR pathway to enhance autophagy. *Autophagy* (2010) 6(2):239–47. doi: 10.4161/auto.6.2.11062
34. Di Nardo A, Wertz MH, Kwiatkowski E, Tsai PT, Leech JD, Greene-Colozzi E, et al. Neuronal Tsc1/2 complex controls autophagy through AMPK-dependent regulation of ULK1. *Hum Mol Genet* (2014) 23(14):3865–74. doi: 10.1093/hmg/ddu101
35. Galluzzi L, Pietrocola F, Bravo-San Pedro JM, Amaravadi RK, Baehrecke EH, Cecconi F, et al. Autophagy in malignant transformation and cancer progression. *EMBO J* (2015) 34(7):856–80. doi: 10.15252/embj.201490784
36. Janku F, McConkey DJ, Hong DS, Kurzrock R. Autophagy as a target for anticancer therapy. *Nat Rev Clin Oncol* (2011) 8(9):528–39. doi: 10.1038/nrclinonc.2011.71
37. Kotani T, Kirisako H, Koizumi M, Ohsumi Y, Nakatogawa H. The Atg2-Atg18 complex tethers pre-autophagosomal membranes to the endoplasmic reticulum for autophagosome formation. *Proc Natl Acad Sci* (2018) 115(41):10363–8. doi: 10.1073/pnas.1806727115
38. Ktistakis NT, Tooze SA. Digesting the Expanding Mechanisms of Autophagy. *Trends Cell Biol* (2016) 26(8):624–35. doi: 10.1016/j.tcb.2016.03.006
39. Ge L, Melville D, Zhang M, Schekman R. The ER–Golgi intermediate compartment is a key membrane source for the LC3 lipidation step of autophagosome biogenesis. *Elife* (2013) 2:1–23. doi: 10.7554/eLife.00947
40. Wei Y, Liu M, Li X, Liu J, Li H. Origin of the Autophagosome Membrane in Mammals. *BioMed Res Int* (2018) 2018:1–9. doi: 10.1155/2018/1012789
41. Lamb CA, Yoshimori T, Tooze SA. The autophagosome: origins unknown, biogenesis complex. *Nat Rev Mol Cell Biol* (2013) 14(12):759–74. doi: 10.1038/nrm3696
42. Ge L, Baskaran S, Schekman R, Hurley JH. The protein-vesicle network of autophagy. *Curr Opin Cell Biol* (2014) 29(1):18–24. doi: 10.1016/j.ceb.2014.02.005
43. Decuypere JP, Parys JB, Bultynck G. Regulation of the Autophagic Bcl-2/Beclin 1 Interaction. *Cells* (2012) 1(3):284–312. doi: 10.3390/cells1030284
44. Wen J, Mai Z, Zhao M, Wang X, Chen T. Full anti-apoptotic function of Bcl-XL complexed with Beclin-1 verified by live-cell FRET assays. *Biochem Biophys Res Commun* (2019) 511(3):700–4. doi: 10.1016/j.bbrc.2019.02.107
45. Nakajima S, Aikawa C, Nozawa T, Minowa-Nozawa A, Toh H, Nakagawa I. Bcl-xL Affects Group A Streptococcus-Induced Autophagy Directly, by Inhibiting Fusion between Autophagosomes and Lysosomes, and Indirectly, by Inhibiting Bacterial Internalization via Interaction with Beclin 1-UVRAG. Trajkovic V, ed. *PloS One* (2017) 12(1):e0170138. doi: 10.1371/journal.pone.0170138

46. Into T, Inomata M, Takayama E, Takigawa T. Autophagy in regulation of Toll-like receptor signaling. *Cell Signal* (2012) 24(6):1150–62. doi: 10.1016/j.cellsig.2012.01.020
47. Marquez RT, Xu L. Bcl-2:Beclin 1 complex: multiple, mechanisms regulating autophagy/apoptosis toggle switch. *Am J Cancer Res* (2012) 2(2):214–21. doi: 2156-6976/ajcr0000098ajcr0000098
48. Grasso D, Ropolo A, Vaccaro MI. Autophagy in Cell Fate and Diseases. *Cell Death - Autophagy, Apoptosis and Necrosis* (2015) 1:3–26. doi: 10.5772/61553
49. Fujita N, Itoh T, Omori H, Fukuda M, Noda T, Yoshimori T. The Atg16L Complex Specifies the Site of LC3 Lipidation for Membrane Biogenesis in Autophagy. Riezman H, ed. *Mol Biol Cell* (2008) 19(5):2092–100. doi: 10.1091/mbc.e07-12-1257
50. Winer H, Fraiberg M, Abada A, Dadosh T, Tamim-Yecheskel BC, Elazar Z. Autophagy differentially regulates TNF receptor Fn14 by distinct mammalian Atg8 proteins. *Nat Commun* (2018) 9(1):3744. doi: 10.1038/s41467-018-06275-1
51. Schaaf MBE, Keulers TG, Vooijs MA, Rouschop KMA. LC3/GABARAP family proteins: autophagy-(un)related functions. *FASEB J* (2016) 30(12):3961–78. doi: 10.1096/fj.201600698R
52. Brier LW, Ge L, Stjepanovic G, Thelen AM, Hurley J, Schekman R. Regulation of LC3 Lipidation by the Autophagy-Specific Class III Phosphatidylinositol-3 Kinase Complex. *Mol Biol Cell* (2019) 30(9):1051–128. doi: 10.1091/mbc.E18-11-0743
53. Moscat J, Karin M, Diaz-Meco MT. p62 in Cancer: Signaling Adaptor Beyond Autophagy. *Cell* (2016) 167(3):606–9. doi: 10.1016/j.cell.2016.09.030
54. Lau A, Wang XJ, Zhao F, Villeneuve NF, Wu T, Jiang T, et al. A Noncanonical Mechanism of Nrf2 Activation by Autophagy Deficiency: Direct Interaction between Keap1 and p62. *Mol Cell Biol* (2010) 30(13):3275–85. doi: 10.1128/MCB.00248-10
55. Valencia T, Kim JY, Abu-Baker S, Moscat-Pardos J, Ahn CS, Reina-Campos M, et al. Metabolic Reprogramming of Stromal Fibroblasts through p62-mTORC1 Signaling Promotes Inflammation and Tumorigenesis. *Cancer Cell* (2014) 26(1):121–35. doi: 10.1016/j.ccr.2014.05.004
56. Zhao YG, Zhang H. Autophagosome maturation: An epic journey from the ER to lysosomes. *J Cell Biol* (2019) 218(3):757–70. doi: 10.1083/jcb.201810099
57. Parzych KR, Klionsky DJ. An Overview of Autophagy: Morphology, Mechanism, and Regulation. *Antioxid Redox Signal* (2014) 20(3):460–73. doi: 10.1089/ars.2013.5371
58. Jager S. Role for Rab7 in maturation of late autophagic vacuoles. *J Cell Sci* (2004) 117(20):4837–48. doi: 10.1242/jcs.01370
59. Itakura E, Mizushima N. Characterization of autophagosome formation site by a hierarchical analysis of mammalian Atg proteins. *Autophagy* (2010) 6(6):764–76. doi: 10.4161/auto.6.6.12709
60. Gatica D, Lahiri V, Klionsky DJ. Cargo recognition and degradation by selective autophagy. *Nat Cell Biol* (2018) 20(3):233–42. doi: 10.1038/s41556-018-0037-z
61. Jing K, Lim K. Why is autophagy important in human diseases? *Exp Mol Med* (2012) 44(2):69. doi: 10.3858/emm.2012.44.2.028
62. Yang Y, Klionsky DJ. Autophagy and disease: unanswered questions. *Cell Death Differ* (2020) 27(3):858–71. doi: 10.1038/s41418-019-0480-9
63. Zachari M, Gudmundsson SR, Li Z, Manifava M, Shah R, Smith M, et al. Selective Autophagy of Mitochondria on a Ubiquitin-Endoplasmic-Reticulum Platform. *Dev Cell* (2019) 50(5):627–43.e5. doi: 10.1016/j.devcel.2019.06.016
64. Germain K, Kim PK. Pexophagy: A Model for Selective Autophagy. *Int J Mol Sci* (2020) 21(2):578. doi: 10.3390/ijms21020578
65. Cebollero E, Reggiori F, Kraft C. Reticulophagy and Ribophagy: Regulated Degradation of Protein Production Factories. *Int J Cell Biol* (2012) 2012:1–9. doi: 10.1155/2012/182834
66. Nakatogawa H, Mochida K. Reticulophagy and nucleophagy: New findings and unsolved issues. *Autophagy* (2015) 11(12):2377–8. doi: 10.1080/15548627.2015.1106665
67. Singh R, Cuervo AM. Lipophagy: Connecting Autophagy and Lipid Metabolism. *Int J Cell Biol* (2012) 2012:1–12. doi: 10.1155/2012/282041
68. Bauckman KA, Owusu-Boaitey N, Mysorekar IU. Selective autophagy: Xenophagy. *Methods* (2015) 75:120–7. doi: 10.1016/j.ymeth.2014.12.005
69. Lamark T, Johansen T. Aggrephagy: Selective Disposal of Protein Aggregates by Macroautophagy. *Int J Cell Biol* (2012) 2012:1–21. doi: 10.1155/2012/736905
70. Doherty J, Baehrecke EH. Life, death and autophagy. *Nat Cell Biol* (2018) 20(10):1110–7. doi: 10.1038/s41556-018-0201-5
71. Nah J, Zablocki D, Sadoshima J. Autosis. *JACC Basic to Transl Sci* (2020) 5(8):857–69. doi: 10.1016/j.jacbts.2020.04.014
72. Swart C, Du Toit A, Loos B. Autophagy and the invisible line between life and death. *Eur J Cell Biol* (2016) 95(12):598–610. doi: 10.1016/j.ejcb.2016.10.005
73. Kriel J, Loos B. The good, the bad and the autophagosome: exploring unanswered questions of autophagy-dependent cell death. *Cell Death Differ* (2019) 26(4):640–52. doi: 10.1038/s41418-018-0267-4
74. Sridhar S, Botbol Y, MacIan F, Cuervo AM. Autophagy and disease: Always two sides to a problem. *J Pathol* (2012) 226(2):255–73. doi: 10.1002/path.3025
75. Santana-Codina N, Mancias JD, Kimmelman AC. The Role of Autophagy in Cancer. *Annu Rev Cancer Biol* (2017) 1(1):19–39. doi: 10.1146/annurev-cancerbio-041816-122338
76. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell* (2011) 144(5):646–74. doi: 10.1016/j.cell.2011.02.013
77. Choi KS. Autophagy and cancer. *Exp Mol Med* (2012) 44(2):109. doi: 10.3858/emm.2012.44.2.033
78. Das CK, Mandal M, Kögel D. Pro-survival autophagy and cancer cell resistance to therapy. *Cancer Metastasis Rev* (2018) 37(4):749–66. doi: 10.1007/s10555-018-9727-z
79. Singh SS, Vats S, Chia AY-Q, Tan TZ, Deng S, Ong MS, et al. Dual role of autophagy in hallmarks of cancer. *Oncogene* (2018) 37(9):1142–58. doi: 10.1038/s41388-017-0046-6
80. Wang K, Klionsky DJ. Mitochondria removal by autophagy. *Autophagy* (2011) 7(3):297–300. doi: 10.4161/auto.7.3.14502
81. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* (1999) 402(6762):672–6. doi: 10.1038/45257
82. Erlich S, Mizrachy L, Segev O, Lindenboim L, Zmira O, Aldi-Harel S, et al. Differential Interactions Between Beclin 1 and Bcl-2 Family Members. *Autophagy* (2007) 2007:3(6):561–8. doi: 10.4161/auto.4713
83. Kang R, Zeh HJ, Lotze MT, Tang D. The Beclin 1 network regulates autophagy and apoptosis. *Cell Death Differ* (2011) 18(4):571–80. doi: 10.1038/cdd.2010.191
84. Toton E, Lisiak N, Sawicka P, Rybczynska M. Beclin-1 and its role as a target for anticancer therapy. *J Physiol Pharmacol* (2014) 65(4):459–67.
85. Hill SM, Wrobel L, Rubinsztein DC. Post-translational modifications of Beclin 1 provide multiple strategies for autophagy regulation. *Cell Death Differ* (2019) 26(4):617–29. doi: 10.1038/s41418-018-0254-9
86. Ávalos Y, Canales J, Bravo-Sagua R, Criollo A, Lavandero S, Quest AFG. Tumor Suppression and Promotion by Autophagy. *BioMed Res Int* (2014) 2014:1–15. doi: 10.1155/2014/603980
87. Eliopoulos AG, Havaki S, Gorgoulis VG. DNA Damage Response and Autophagy: A Meaningful Partnership. *Front Genet* (2016) 7:204(NOV). doi: 10.3389/fgene.2016.00204
88. Rodriguez-Rocha H, Garcia-Garcia A, Panayiotidis MI, Franco R. DNA damage and autophagy. *Mutat Res Mol Mech Mutagen* (2011) 711(1-2):158–66. doi: 10.1016/j.mrfmmm.2011.03.007
89. Yun C, Lee S. The Roles of Autophagy in Cancer. *Int J Mol Sci* (2018) 19(11):3466. doi: 10.3390/ijms19113466
90. Takamura A, Komatsu M, Hara T, Sakamoto A, Kishi C, Waguri S, et al. Autophagy-deficient mice develop multiple liver tumors. *Genes Dev* (2011) 25(8):795–800. doi: 10.1101/gad.2016211
91. Kimmelman AC. The dynamic nature of autophagy in cancer. *Genes Dev* (2011) 25(19):1999–2010. doi: 10.1101/gad.17558811
92. Mariño G, Salvador-Montoliu N, Fueyo A, Knecht E, Mizushima N, López-Otín C. Tissue-specific Autophagy Alterations and Increased Tumorigenesis in Mice Deficient in Atg4C/Autophagin-3. *J Biol Chem* (2007) 282(25):18573–83. doi: 10.1074/jbc.M701194200
93. Cianfanelli V, Fuoco C, Lorente M, Salazar M, Quondamatteo F, Gherardini PF, et al. AMBRA1 links autophagy to cell proliferation and tumorigenesis by promoting c-Myc dephosphorylation and degradation. *Nat Cell Biol* (2015) 17(1):20–30. doi: 10.1038/ncb3072

94. Maiuri MC, Galluzzi L, Morselli E, Kepp O, Malik SA, Kroemer G. Autophagy regulation by p53. *Curr Opin Cell Biol* (2010) 22(2):181–5. doi: 10.1016/j.ceb.2009.12.001
95. Luo Z, Zang M, Guo W. AMPK as a metabolic tumor suppressor: control of metabolism and cell growth. *Futur Oncol* (2010) 6(3):457–70. doi: 10.2217/fon.09.174
96. Tasdemir E, Maiuri MC, Morselli E, Criollo A, D'Amelio M, Djavaheri-Mergny M, et al. A dual role of p53 in the control of autophagy. *Autophagy* (2008) 4(6):810–4. doi: 10.4161/auto.6486
97. Mrakovcic M, Fröhlich LF. p53-Mediated Molecular Control of Autophagy in Tumor Cells. *Biomolecules* (2018) 8(2):14. doi: 10.3390/biom8020014
98. Rosenfeldt MT, O'Prey J, Morton JP, Nixon C, MacKay G, Mrowinska A, et al. p53 status determines the role of autophagy in pancreatic tumour development. *Nature* (2013) 504(7479):296–300. doi: 10.1038/nature12865
99. White E. Autophagy and p53. *Cold Spring Harb Perspect Med* (2016) 6(4):a026120. doi: 10.1101/cshperspect.a026120
100. Kenzelmann Broz D, Spano Mello S, Bieging KT, Jiang D, Dusek RL, Brady CA, et al. Global genomic profiling reveals an extensive p53-regulated autophagy program contributing to key p53 responses. *Genes Dev* (2013) 27(9):1016–31. doi: 10.1101/gad.212282.112
101. Eby KG, Rosenbluth JM, Mays DJ, Marshall CB, Barton CE, Sinha S, et al. ISG20L1 is a p53 family target gene that modulates genotoxic stress-induced autophagy. *Mol Cancer* (2010) 9(1):95. doi: 10.1186/1476-4598-9-95
102. Mowers EE, Sharifi MN, Macleod KF. Autophagy in cancer metastasis. *Oncogene* (2017) 36(12):1619–30. doi: 10.1038/onc.2016.333
103. Yang ZJ, Chee CE, Huang S, Sinicrope FA. The Role of Autophagy in Cancer: Therapeutic Implications. *Mol Cancer Ther* (2011) 10(9):1533–41. doi: 10.1158/1535-7163.MCT-11-0047
104. Simon T, Gagliano T, Giamas G. Direct Effects of Anti-Angiogenic Therapies on Tumor Cells: VEGF Signaling. *Trends Mol Med* (2017) 23(3):282–92. doi: 10.1016/j.molmed.2017.01.002
105. Salabei JK, Cummins TD, Singh M, Jones SP, Bhatnagar A, Hill BG. PDGF-mediated autophagy regulates vascular smooth muscle cell phenotype and resistance to oxidative stress. *Biochem J* (2013) 451(3):375–88. doi: 10.1042/BJ20121344
106. Bharath LP, Cho JM, Park S-K, Ruan T, Li Y, Mueller R, et al. Endothelial Cell Autophagy Maintains Shear Stress–Induced Nitric Oxide Generation via Glycolysis-Dependent Purinergic Signaling to Endothelial Nitric Oxide Synthase. *Arterioscler Thromb Vasc Biol* (2017) 37(9):1646–56. doi: 10.1161/ATVBAHA.117.309510
107. Qu X. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest* (2003) 112(12):1809–20. doi: 10.1172/JCI200320039
108. Das CK, Banerjee I, Mandal M. Pro-survival autophagy: An emerging candidate of tumor progression through maintaining hallmarks of cancer. *Semin Cancer Biol* (2019) 66:59–74. doi: 10.1016/j.semcancer.2019.08.020
109. Bykov VJN, Eriksson SE, Bianchi J, Wiman KG. Targeting mutant p53 for efficient cancer therapy. *Nat Rev Cancer* (2018) 18(2):89–102. doi: 10.1038/nrc.2017.109
110. Huo Y, Cai H, Teplova I, Bowman-Colin C, Chen G, Price S, et al. Autophagy Opposes p53-Mediated Tumor Barrier to Facilitate Tumorigenesis in a Model of PALB2-Associated Hereditary Breast Cancer. *Cancer Discov* (2013) 3(8):894–907. doi: 10.1158/2159-8290.CD-13-0011
111. Moscat J, Diaz-Meco MT. p62: a versatile multitasker takes on cancer. *Trends Biochem Sci* (2012) 37(6):230–6. doi: 10.1016/j.tibs.2012.02.008
112. Li L, Shen C, Nakamura E, Ando K, Signoretti S, Beroukhim R, et al. SQSTM1 Is a Pathogenic Target of 5q Copy Number Gains in Kidney Cancer. *Cancer Cell* (2013) 24(6):738–50. doi: 10.1016/j.ccr.2013.10.025
113. Umemura A, He F, Taniguchi K, Nakagawa H, Yamachika S, font-Burgada J, et al. p62, Upregulated during Preneoplasia, Induces Hepatocellular Carcinogenesis by Maintaining Survival of Stressed HCC-Initiating Cells. *Cancer Cell* (2016) 29(6):935–48. doi: 10.1016/j.ccell.2016.04.006
114. Zhang J, Yang S, Xu B, Wang T, Zheng Y, Liu F, et al. p62 functions as an oncogene in colorectal cancer through inhibiting apoptosis and promoting cell proliferation by interacting with the vitamin D receptor. *Cell Prolif* (2019) 52(3):e12585. doi: 10.1111/cpr.12585
115. Ren F, Shu G, Liu G, Liu D, Zhou J, Yuan L, et al. Knockdown of p62/sequestosome 1 attenuates autophagy and inhibits colorectal cancer cell growth. *Mol Cell Biochem* (2014) 385(1-2):95–102. doi: 10.1007/s11010-013-1818-0
116. Schmukler E, Kloog Y, Pinkas-Kramarski R. Ras and autophagy in cancer development and therapy. *Oncotarget* (2014) 5(3):577–86. doi: 10.18632/oncotarget.1775
117. Lock R, Kenific CM, Leidal AM, Salas E, Debnath J. Autophagy-dependent production of secreted factors facilitates oncogenic RAS-Driven invasion. *Cancer Discov* (2014) 4(4):466–79. doi: 10.1158/2159-8290.CD-13-0841
118. Guo JY, Karsli-Uzunbas G, Mathew R, Aisner SC, Kamphorst JJ, Strohecker AM, et al. Autophagy suppresses progression of K-ras-induced lung tumors to oncocytomas and maintains lipid homeostasis. *Genes Dev* (2013) 27(13):1447–61. doi: 10.1101/gad.219642.113
119. Ling J, Kang Y, Zhao R, Xia Q, Lee D-F, Chang Z, et al. KrasG12D-Induced IKK2/β/NF-κB Activation by IL-1α and p62 Feedforward Loops Is Required for Development of Pancreatic Ductal Adenocarcinoma. *Cancer Cell* (2012) 21(1):105–20. doi: 10.1016/j.ccr.2011.12.006
120. Sosa MS, Bragado P, Debnath J, Aguirre-Ghiso JA. Regulation of Tumor Cell Dormancy by Tissue Microenvironments and Autophagy. In: H Enderling, N Almog and L Hlatky, editors. *Advances in Experimental Medicine and Biology*, vol. 734 . New York, NY: Springer New York (2013). p. 73–89. doi: 10.1007/978-1-4614-1445-2_5
121. Lorin S, Hamaï A, Mehrpour M, Codogno P. Autophagy regulation and its role in cancer. *Semin Cancer Biol* (2013) 23(5):361–79. doi: 10.1016/j.semcancer.2013.06.007
122. Paoli P, Giannoni E, Chiarugi P. Anoikis molecular pathways and its role in cancer progression. *Biochim Biophys Acta - Mol Cell Res* (2013) 1833(12):3481–98. doi: 10.1016/j.bbamcr.2013.06.026
123. Buchheit CL, Weigel KJ, Schafer ZT. Cancer cell survival during detachment from the ECM: multiple barriers to tumour progression. *Nat Rev Cancer* (2014) 14(9):632–41. doi: 10.1038/nrc3789
124. Zhi X, Zhong Q. Autophagy in cancer. *F1000Prime Rep* (2015) 7 (February):1–12. doi: 10.12703/P7-18
125. Swartz MA, Iida N, Roberts EW, Sangaletti S, Wong MH, Yull FE, et al. Tumor Microenvironment Complexity: Emerging Roles in Cancer Therapy: Figure 1. *Cancer Res* (2012) 72(10):2473–80. doi: 10.1158/0008-5472.CAN-12-0122
126. Camuzard O, Santucci-Darmanin S, Carle GF, Pierrefite-Carle V. Autophagy in the crosstalk between tumor and microenvironment. *Cancer Lett* (2020) 490:143–53. doi: 10.1016/j.canlet.2020.06.015
127. Roma-Rodrigues C, Mendes R, Baptista P, Fernandes A. Targeting Tumor Microenvironment for Cancer Therapy. *Int J Mol Sci* (2019) 20(4):840. doi: 10.3390/ijms20040840
128. Ye J, Wu D, Wu P, Chen Z, Huang J. The cancer stem cell niche: cross talk between cancer stem cells and their microenvironment. *Tumor Biol* (2014) 35(5):3945–51. doi: 10.1007/s13277-013-1561-x
129. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer* (2006) 6 (5):392–401. doi: 10.1038/nrc1877
130. Folkerts H, Hilgendorf S, Vellenga E, Bremer E, Wiersma VR. The multifaceted role of autophagy in cancer and the microenvironment. *Med Res Rev* (2019) 39(2):517–60. doi: 10.1002/med.21531
131. Guo JY, Teng X, Laddha SV, Ma S, Van Nostrand SC, Yang Y, et al. Autophagy provides metabolic substrates to maintain energy charge and nucleotide pools in Ras-driven lung cancer cells. *Genes Dev* (2016) 30 (15):1704–17. doi: 10.1101/gad.283416.116
132. Ngabire D, Kim G-D. Autophagy and Inflammatory Response in the Tumor Microenvironment. *Int J Mol Sci* (2017) 18(9):2016. doi: 10.3390/ijms18092016
133. Hui L, Chen Y. Tumor microenvironment: Sanctuary of the devil. *Cancer Lett* (2015) 368(1):7–13. doi: 10.1016/j.canlet.2015.07.039
134. Anthony BA, Link DC. Regulation of hematopoietic stem cells by bone marrow stromal cells. *Trends Immunol* (2014) 35(1):32–7. doi: 10.1016/j.it.2013.10.002
135. Guan J-L, Simon AK, Prescott M, Menendez JA, Liu F, Wang F, et al. Autophagy in stem cells. *Autophagy* (2013) 9(6):830–49. doi: 10.4161/auto.24132
136. Ito K, Ito K. Hematopoietic stem cell fate through metabolic control. *Exp Hematol* (2018) 64:1–11. doi: 10.1016/j.exphem.2018.05.005

137. Salemi S, Yousefi S, Constantinescu MA, Fey MF, Simon H-U. Autophagy is required for self-renewal and differentiation of adult human stem cells. *Cell Res* (2012) 22(2):432–5. doi: 10.1038/cr.2011.200
138. Liu Q, Chen L, Atkinson JM, Claxton DF, Wang H-G. Atg5-dependent autophagy contributes to the development of acute myeloid leukemia in an MLL-AF9-driven mouse model. *Cell Death Dis* (2016) 7(9):e2361–1. doi: 10.1038/cddis.2016.264
139. Jacquel A, Obba S, Boyer L, Dufies M, Robert G, Gounon P, et al. Autophagy is required for CSF-1–induced macrophagic differentiation and acquisition of phagocytic functions. *Blood* (2012) 119(19):4527–31. doi: 10.1182/blood-2011-11-392167
140. Jacquel A, Benikhlef N, Paggetti J, Lalaoui N, Guery L, Dufour EK, et al. Colony-stimulating factor-1–induced oscillations in phosphatidylinositol-3 kinase/AKT are required for caspase activation in monocytes undergoing differentiation into macrophages. *Blood* (2009) 114(17):3633–41. doi: 10.1182/blood-2009-03-208843
141. Zhang Y, Morgan MJ, Chen K, Choksi S, Liu Z. Induction of autophagy is essential for monocyte-macrophage differentiation. *Blood* (2012) 119 (12):2895–905. doi: 10.1182/blood-2011-08-372383
142. Modi J, Menzie-Suderam J, Xu H, Trujillo P, Medley K, Marshall ML, et al. Mode of action of granulocyte-colony stimulating factor (G-CSF) as a novel therapy for stroke in a mouse model. *J BioMed Sci* (2020) 27(1):19. doi: 10.1186/s12929-019-0597-7
143. Martinez-Outschoorn UE, Whitaker-Menezes D, Lin Z, Flomenberg N, Howell A, Pestell RG, et al. Cytokine production and inflammation drive autophagy in the tumor microenvironment. *Cell Cycle* (2011) 10(11):1784–93. doi: 10.4161/cc.10.11.15674
144. Goswami KK, Ghosh T, Ghosh S, Sarkar M, Bose A, Baral R. Tumor promoting role of anti-tumor macrophages in tumor microenvironment. *Cell Immunol* (2017) 316(April):1–10. doi: 10.1016/j.cellimm.2017.04.005
145. Martinez-Outschoorn UE, Trimmer C, Lin Z, Whitaker-Menezes D, Chiavarina B, Zhou J, et al. Autophagy in cancer associated fibroblasts promotes tumor cell survival. *Cell Cycle* (2010) 9(17):3515–33. doi: 10.4161/cc.9.17.12928
146. Capparelli C, Guido C, Whitaker-Menezes D, Bonuccelli G, Balliet R, Pestell TG, et al. Autophagy and senescence in cancer-associated fibroblasts metabolically supports tumor growth and metastasis, via glycolysis and ketone production. *Cell Cycle* (2012) 11(12):2285–302. doi: 10.4161/cc.20718
147. Grasso D, Garcia MN, Iovanna JL. Autophagy in Pancreatic Cancer. *Int J Cell Biol* (2012) 2012:1–7. doi: 10.1155/2012/760498
148. Mowers EE, Sharifi MN, Macleod KF. Functions of autophagy in the tumor microenvironment and cancer metastasis. *FEBS J* (2018) 285(10):1751–66. doi: 10.1111/febs.14388
149. Endo S, Nakata K, Ohuchida K, Takesue S, Nakayama H, Abe T, et al. Autophagy Is Required for Activation of Pancreatic Stellate Cells, Associated With Pancreatic Cancer Progression and Promotes Growth of Pancreatic Tumors in Mice. *Gastroenterology* (2017) 152(6):1492–1506.e24. doi: 10.1053/j.gastro.2017.01.010
150. Schaaf MB, Houbaert D, Meçe O, Agostinis P. Autophagy in endothelial cells and tumor angiogenesis. *Cell Death Differ* (2019) 26(4):665–79. doi: 10.1038/s41418-019-0287-8
151. Welti J, Loges S, Dimmeler S, Carmeliet P. Recent molecular discoveries in angiogenesis and antiangiogenic therapies in cancer. *J Clin Invest* (2013) 123 (8):3190–200. doi: 10.1172/JCI70212
152. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* (2000) 407(6801):249–57. doi: 10.1038/35025220
153. Filippi I, Saltarella I, Aldinucci C, Carraro F, Ria R, Vacca A, et al. Different Adaptive Responses to Hypoxia in Normal and Multiple Myeloma Endothelial Cells. *Cell Physiol Biochem* (2018) 46(1):203–12. doi: 10.1159/000488423
154. Chandra A, Rick J, Yagnik G, Aghi MK. Autophagy as a mechanism for anti-angiogenic therapy resistance. *Semin Cancer Biol* (2020) 66(January):75–88. doi: 10.1016/j.semcancer.2019.08.031
155. Maes H, Olmeda D, Soengas MS, Agostinis P. Vesicular trafficking mechanisms in endothelial cells as modulators of the tumor vasculature and targets of antiangiogenic therapies. *FEBS J* (2016) 283(1):25–38. doi: 10.1111/febs.13545
156. Maes H, Rubio N, Garg AD, Agostinis P. Autophagy: shaping the tumor microenvironment and therapeutic response. *Trends Mol Med* (2013) 19 (7):428–46. doi: 10.1016/j.molmed.2013.04.005
157. Ma Y, Galluzzi L, Zitvogel L, Kroemer G. Autophagy and Cellular Immune Responses. *Immunity* (2013) 39(2):211–27. doi: 10.1016/j.immuni.2013.07.017
158. Caron E, Vincent K, Fortier M, Laverdure J-P, Bramoullé A, Hardy M-P, et al. The MHC I immunopeptidome conveys to the cell surface an integrative view of cellular regulation. *Mol Syst Biol* (2011) 7(1):533. doi: 10.1038/msb.2011.68
159. Michaud M, Martins I, Sukkurwala AQ, Adjemian S, Ma Y, Pellegatti P, et al. Autophagy-Dependent Anticancer Immune Responses Induced by Chemotherapeutic Agents in Mice. *Science (80-)* (2011) 334(6062):1573–7. doi: 10.1126/science.1208347
160. Wildenberg ME, Vos ACW, Wolfkamp SCS, Duijvestein M, Verhaar AP, Te Velde AA, et al. Autophagy Attenuates the Adaptive Immune Response by Destabilizing the Immunologic Synapse. *Gastroenterology* (2012) 142 (7):1493–503.e6. doi: 10.1053/j.gastro.2012.02.034
161. Jia W, He Y-W. Temporal Regulation of Intracellular Organelle Homeostasis in T Lymphocytes by Autophagy. *J Immunol* (2011) 186(9):5313–22. doi: 10.4049/jimmunol.1002404
162. Fiegl D, Kägebein D, Liebler-Tenorio EM, Weisser T, Sens M, Gutjahr M, et al. Amphisomal Route of MHC Class I Cross-Presentation in Bacteria-Infected Dendritic Cells. *J Immunol* (2013) 190(6):2791–806. doi: 10.4049/jimmunol.1202741
163. Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ. Natural Innate and Adaptive Immunity to Cancer. *Annu Rev Immunol* (2011) 29(1):235–71. doi: 10.1146/annurev-immunol-031210-101324
164. Lapaquette P, Guzzo J, Bretillon L, Bringer M-A. Cellular and Molecular Connections between Autophagy and Inflammation. *Mediators Inflamm* (2015) 2015:1–13. doi: 10.1155/2015/398483
165. Vidya MK, Kumar VG, Sejian V, Bagath M, Krishnan G, Bhatta R. Toll-like receptors: Significance, ligands, signaling pathways, and functions in mammals. *Int Rev Immunol* (2018) 37(1):20–36. doi: 10.1080/08830185.2017.1380200
166. Hontelez S, Sanecka A, Netea MG, van Spriel AB, Adema GJ. Molecular view on PRR cross-talk in antifungal immunity. *Cell Microbiol* (2012) 14(4):467–74. doi: 10.1111/j.1462-5822.2012.01748.x
167. Sharma V, Verma S, Seranova E, Sarkar S, Kumar D. Selective Autophagy and Xenophagy in Infection and Disease. *Front Cell Dev Biol* (2018) 6:147 (NOV). doi: 10.3389/fcell.2018.00147
168. Delgado MA, Elmaoued RA, Davis AS, Kyei G, Deretic V. Toll-like receptors control autophagy. *EMBO J* (2008) 27(7):1110–21. doi: 10.1038/emboj.2008.31
169. Shi C-S, Kehrl JH. MyD88 and Trif Target Beclin 1 to Trigger Autophagy in Macrophages. *J Biol Chem* (2008) 283(48):33175–82. doi: 10.1074/jbc.M804478200
170. Weichhart T, Hengstschläger M, Linke M. Regulation of innate immune cell function by mTOR. *Nat Rev Immunol* (2015) 15(10):599–614. doi: 10.1038/nri3901
171. Hamon MA, Quintin J. Innate immune memory in mammals. *Semin Immunol* (2016) 28(4):351–8. doi: 10.1016/j.smim.2016.05.003
172. Mah LY, Ryan KM. Autophagy and Cancer. *Cold Spring Harb Perspect Biol* (2012) 4(1):a008821–a008821. doi: 10.1101/cshperspect.a008821
173. Wieczorek M, Abualrous ET, Sticht J, Álvalo-Benito M, Stolzenberg S, Noé F, et al. Major Histocompatibility Complex (MHC) Class I and MHC Class II Proteins: Conformational Plasticity in Antigen Presentation. *Front Immunol* (2017) 8:292(MAR). doi: 10.3389/fimmu.2017.00292
174. Li B, Lei Z, Lichty BD, Li D, Zhang G-M, Feng Z-H, et al. Autophagy facilitates major histocompatibility complex class I expression induced by IFN-γ in B16 melanoma cells. *Cancer Immunol Immunother* (2010) 59 (2):313–21. doi: 10.1007/s00262-009-0752-1
175. Crotzer VL, Blum JS. Autophagy and adaptive immunity. *Immunology* (2010) 131(1):no–o. doi: 10.1111/j.1365-2567.2010.03321.x
176. You L, Jin S, Zhu L, Qian W. Autophagy, autophagy-associated adaptive immune responses and its role in hematologic malignancies. *Oncotarget* (2017) 8(7):12374–88. doi: 10.18632/oncotarget.13583

177. Puleston DJ, Simon AK. Autophagy in the immune system. *Immunology* (2014) 141(1):1–8. doi: 10.1111/imm.12165
178. Mortensen M, Ferguson DJP, Edelmann M, Kessler B, Morten KJ, Komatsu M, et al. Loss of autophagy in erythroid cells leads to defective removal of mitochondria and severe anemia in vivo. *Proc Natl Acad Sci* (2010) 107 (2):832–7. doi: 10.1073/pnas.0913170107
179. Miller BC, Zhao Z, Stephenson LM, Cadwell K, Pua HH, Lee HK, et al. The autophagy gene ATG5 plays an essential role in B lymphocyte development. *Autophagy* (2008) 4(3):309–14. doi: 10.4161/auto.5474
180. Rybstein MD, Bravo-San Pedro JM, Kroemer G, Galluzzi L. The autophagic network and cancer. *Nat Cell Biol* (2018) 20(3):243–51. doi: 10.1038/s41556-018-0042-2
181. Monkkonen T, Debnath J. Inflammatory signaling cascades and autophagy in cancer. *Autophagy* (2018) 14(2):190–8. doi: 10.1080/15548627.2017.1345412
182. Nakamura K, Smyth MJ. Targeting cancer-related inflammation in the era of immunotherapy. *Immunol Cell Biol* (2017) 95(4):325–32. doi: 10.1038/icb.2016.126
183. Deretic V, Jiang S, Dupont N. Autophagy intersections with conventional and unconventional secretion in tissue development, remodeling and inflammation. *Trends Cell Biol* (2012) 22(8):397–406. doi: 10.1016/j.tcb.2012.04.008
184. Nawas AF, Mistry R, Narayanan S, Thomas-Jardin SE, Ramachandran J, Ravichandran J, et al. IL-1 induces p62/SQSTM1 and autophagy in ERα + /PR + BCa cell lines concomitant with ERα and PR repression, conferring an ERα – /PR – BCa-like phenotype. *J Cell Biochem* (2019) 120(2):1477–91. doi: 10.1002/jcb.27340
185. Li TT, Zhu D, Mou T, Guo Z, Pu JL, Chen QS, et al. IL-37 induces autophagy in hepatocellular carcinoma cells by inhibiting the PI3K/AKT/mTOR pathway. *Mol Immunol* (2017) 87(1):132–40. doi: 10.1016/j.molimm.2017.04.010
186. Chio IIC, Tuveson DA. ROS in Cancer: The Burning Question. *Trends Mol Med* (2017) 23(5):411–29. doi: 10.1016/j.molmed.2017.03.004
187. Hoesel B, Schmid JA. The complexity of NF-κB signaling in inflammation and cancer. *Mol Cancer* (2013) 12(1):86. doi: 10.1186/1476-4598-12-86
188. Duran A, Linares JF, Galvez AS, Wikenheiser K, Flores JM, Diaz-Meco MT, et al. The Signaling Adaptor p62 Is an Important NF-κB Mediator in Tumorigenesis. *Cancer Cell* (2008) 13(4):343–54. doi: 10.1016/j.ccr.2008.02.001
189. Schwartz L, Supuran C, Alfarouk K. The Warburg Effect and the Hallmarks of Cancer. *Anticancer Agents Med Chem* (2017) 17(2):164–70. doi: 10.2174/1871520616666161031143301
190. Pavlides S, Tsirigos A, Migneco G, Whitaker-Menezes D, chiavarina B, Flomenberg N, et al. The autophagic tumor stroma model of cancer. *Cell Cycle* (2010) 9(17):3485–505. doi: 10.4161/cc.9.17.12721
191. Xing Y, Zhao S, Zhou BP, Mi J. Metabolic reprogramming of the tumour microenvironment. *FEBS J* (2015) 282(20):3892–8. doi: 10.1111/febs.13402
192. Wei H, Wei S, Gan B, Peng X, Zou W, Guan J-L. Suppression of autophagy by FIP200 deletion inhibits mammary tumorigenesis. *Genes Dev* (2011) 25 (14):1510–27. doi: 10.1101/gad.2051011
193. Karvela M, Baquero P, Kuntz EM, Mukhopadhyay A, Mitchell R, Allan EK, et al. ATG7 regulates energy metabolism, differentiation and survival of Philadelphia-chromosome-positive cells. *Autophagy* (2016) 12(6):936–48. doi: 10.1080/15548627.2016.1162359
194. Correia M, Pinheiro P, Batista R, Soares P, Sobrinho-Simões M, Máximo V. Etiopathogenesis of oncocytomas. *Semin Cancer Biol* (2017) 47:82–94. doi: 10.1016/j.semcancer.2017.06.014
195. Amaravadi R, Kimmelman AC, White E. Recent insights into the function of autophagy in cancer. *Genes Dev* (2016) 30(17):1913–30. doi: 10.1101/gad.287524.116
196. Vara-Perez M, Felipe-Abrio B, Agostinis P. Mitophagy in Cancer: A Tale of Adaptation. *Cells* (2019) 8(5):493. doi: 10.3390/cells8050493
197. Semenza GL. Hypoxia-inducible factor 1: Regulator of mitochondrial metabolism and mediator of ischemic preconditioning. *Biochim Biophys Acta - Mol Cell Res* (2011) 1813(7):1263–8. doi: 10.1016/j.bbamcr.2010.08.006
198. Sowter HM, Ratcliffe PJ, Watson P, Greenberg AH, Harris AL. HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors. *Cancer Res* (2001) 61(18):6669–73.
199. Lyons A, Coleman M, Riis S, Favre C, O'Flanagan CH, Zhdanov AV, et al. Insulin-like growth factor 1 signaling is essential for mitochondrial biogenesis and mitophagy in cancer cells. *J Biol Chem* (2017) 292 (41):16983–98. doi: 10.1074/jbc.M117.792838
200. Agnihotri S, Golbourn B, Huang X, Remke M, Younger S, Cairns RA, et al. PINK1 Is a Negative Regulator of Growth and the Warburg Effect in Glioblastoma. *Cancer Res* (2016) 76(16):4708–19. doi: 10.1158/0008-5472.CAN-15-3079
201. Chang HW, Kim MR, Lee HJ, Lee HJ, Kim GC, Lee YS, et al. p53/BNIP3-dependent mitophagy limits glycolytic shift in radioresistant cancer. *Oncogene* (2019) 38(19):3729–42. doi: 10.1038/s41388-019-0697-6
202. Hoshino A, Ariyoshi M, Okawa Y, Kaimoto S, Uchihashi M, Fukai K, et al. Inhibition of p53 preserves Parkin-mediated mitophagy and pancreatic -cell function in diabetes. *Proc Natl Acad Sci* (2014) 111(8):3116–21. doi: 10.1073/pnas.1318951111
203. Mariño G, Pietrocola F, Eisenberg T, Kong Y, Malik SA, Andryushkova A, et al. Regulation of Autophagy by Cytosolic Acetyl-Coenzyme A. *Mol Cell* (2014) 53(5):710–25. doi: 10.1016/j.molcel.2014.01.016
204. Lee JV, Berry CT, Kim K, Sen P, Kim T, Carrer A, et al. Acetyl-CoA promotes glioblastoma cell adhesion and migration through Ca 2+ –NFAT signaling. *Genes Dev* (2018) 32(7-8):497–511. doi: 10.1101/gad.311027.117
205. Schnittert J, Bansal R, Prakash J. Targeting Pancreatic Stellate Cells in Cancer. *Trends Cancer* (2019) 5(2):128–42. doi: 10.1016/j.trecan.2019.01.001
206. Sousa CM, Biancur DE, Wang X, Halbrook CJ, Sherman MH, Zhang L, et al. Pancreatic stellate cells support tumour metabolism through autophagic alanine secretion. *Nature* (2016) 536(7617):479–83. doi: 10.1038/nature19084
207. Kim J, Gao P, Liu Y-C, Semenza GL, Dang CV. Hypoxia-Inducible Factor 1 and Dysregulated c-Myc Cooperatively Induce Vascular Endothelial Growth Factor and Metabolic Switches Hexokinase 2 and Pyruvate Dehydrogenase Kinase 1. *Mol Cell Biol* (2007) 27(21):7381–93. doi: 10.1128/MCB.00440-07
208. Nagao A, Kobayashi M, Koyasu S, Chow CCT, Harada H. HIF-1-Dependent Reprogramming of Glucose Metabolic Pathway of Cancer Cells and Its Therapeutic Significance. *Int J Mol Sci* (2019) 20(2):238. doi: 10.3390/ijms20020238
209. Dang CV. The Interplay Between MYC and HIF in the Warburg Effect. *Ernst Schering Found Symp Proc* (2008) 8:35–53. doi: 10.1007/2789_2008_088
210. Xiong J, Wang L, Fei X-C, Jiang X-F, Zheng Z, Zhao Y, et al. MYC is a positive regulator of choline metabolism and impedes mitophagy-dependent necroptosis in diffuse large B-cell lymphoma. *Blood Cancer J* (2017) 7(7): e582–2. doi: 10.1038/bcj.2017.61
211. Cianfanelli V, Fuoco C, Lorente M, Salazar M, Quondamatteo F, Gherardini PF, et al. Erratum: Corrigendum: AMBRA1 links autophagy to cell proliferation and tumorigenesis by promoting c-Myc dephosphorylation and degradation. *Nat Cell Biol* (2015) 17(5):706–6. doi: 10.1038/ncb3171
212. GARGINI R, GARCÍA-ESCUDERO V, IZQUIERDO M, WANDOSELL F. Oncogene-mediated tumor transformation sensitizes cells to autophagy induction. *Oncol Rep* (2016) 35(6):3689–95. doi: 10.3892/or.2016.4699
213. Poillet-Perez L, Xie X, Zhan L, Yang Y, Sharp DW, Hu ZS, et al. Autophagy maintains tumour growth through circulating arginine. *Nature* (2018) 563 (7732):569–73. doi: 10.1038/s41586-018-0697-7
214. Saha S, Panigrahi DP, Patil S, Bhutia SK. Autophagy in health and disease: A comprehensive review. *BioMed Pharmacother* (2018) 104(February):485–95. doi: 10.1016/j.biopha.2018.05.007
215. Perot BP, Ingersoll MA, Albert ML. The impact of macroautophagy on CD8 + T-cell-mediated antiviral immunity. *Immunol Rev* (2013) 255(1):40–56. doi: 10.1111/imr.12096
216. Abdoli A, Alirezaei M, Mehrbod P, Forouzanfar F. Autophagy: The multi-purpose bridge in viral infections and host cells. *Rev Med Virol* (2018) 28(4): e1973. doi: 10.1002/rmv.1973
217. Rey-Jurado E, Riedel CA, González PA, Bueno SM, Kalergis AM. Contribution of autophagy to antiviral immunity. *FEBS Lett* (2015) 589 (22):3461–70. doi: 10.1016/j.febslet.2015.07.047
218. Tang D, Kang R, Coyne CB, Zeh HJ, Lotze MT. PAMPs and DAMPs: signal 0s that spur autophagy and immunity. *Immunol Rev* (2012) 249(1):158–75. doi: 10.1111/j.1600-065X.2012.01146.x
219. Wang Z, Li C. Xenophagy in innate immunity: A battle between host and pathogen. *Dev Comp Immunol* (2020) 109(March):103693. doi: 10.1016/j.dci.2020.103693

220. Wu M-F, Chen S-T, Hsieh S-L. Distinct regulation of dengue virus-induced inflammasome activation in human macrophage subsets. *J BioMed Sci* (2013) 20(1):36. doi: 10.1186/1423-0127-20-36
221. Ojha CR, Rodriguez M, Karuppan MKM, Lapierre J, Kashanchi F, El-Hage N. Toll-like receptor 3 regulates Zika virus infection and associated host inflammatory response in primary human astrocytes. Jin D-Y, ed. *PloS One* (2019) 14(2):e0208543. doi: 10.1371/journal.pone.0208543
222. Li Y, Wang L-X, Yang G, Hao F, Urba WJ, Hu H-M. Efficient Cross-presentation Depends on Autophagy in Tumor Cells. *Cancer Res* (2008) 68 (17):6889–95. doi: 10.1158/0008-5472.CAN-08-0161
223. Hanada T, Noda NN, Satomi Y, Ichimura Y, Fujioka Y, Takao T, et al. The Atg12-Atg5 Conjugate Has a Novel E3-like Activity for Protein Lipidation in Autophagy. *J Biol Chem* (2007) 282(52):37298–302. doi: 10.1074/jbc.C700195200
224. Shaw PJ, Lamkanfi M, Kanneganti T-D. NOD-like receptor (NLR) signaling beyond the inflammasome. *Eur J Immunol* (2010) 40(3):624–7. doi: 10.1002/eji.200940211
225. Elinav E, Strowig T, Henao-Mejia J, Flavell RA. Regulation of the Antimicrobial Response by NLR Proteins. *Immunity* (2011) 34(5):665–79. doi: 10.1016/j.immuni.2011.05.007
226. Caruso R, Warner N, Inohara N, Núñez G. NOD1 and NOD2: Signaling, Host Defense, and Inflammatory Disease. *Immunity* (2014) 41(6):898–908. doi: 10.1016/j.immuni.2014.12.010
227. Philpott DJ, Sorbara MT, Robertson SJ, Croitoru K, Girardin SE. NOD proteins: regulators of inflammation in health and disease. *Nat Rev Immunol* (2014) 14(1):9–23. doi: 10.1038/nri3565
228. Xi J, Yan M, Li S, Song H, Liu L, Shen Z, et al. NOD1 activates autophagy to aggravate hepatic ischemia-reperfusion injury in mice. *J Cell Biochem* (2019) 120(6):10605–12. doi: 10.1002/jcb.28349
229. Negroni A, Colantoni E, Vitali R, Palone F, Pierdomenico M, Constanzo M, et al. NOD2 induces autophagy to control AIEC bacteria infectiveness in intestinal epithelial cells. *Inflamm Res* (2016) 65(10):803–13. doi: 10.1007/s00011-016-0964-8
230. Osorio F, Reis e Sousa C. Myeloid C-type Lectin Receptors in Pathogen Recognition and Host Defense. *Immunity* (2011) 34(5):651–64. doi: 10.1016/j.immuni.2011.05.001
231. Pahari S, Negi S, Aqdas M, Arnett E, Schlesinger LS, Agrewala JN. Induction of autophagy through CLEC4E in combination with TLR4: an innovative strategy to restrict the survival of Mycobacterium tuberculosis. *Autophagy* (2020) 16(6):1021–43. doi: 10.1080/15548627.2019.1658436
232. Rodriguez KR, Bruns AM, Horvath CM. MDA5 and LGP2: Accomplices and Antagonists of Antiviral Signal Transduction. *J Virol* (2014) 88(15):8194–200. doi: 10.1128/JVI.00640-14
233. Subramani S, Malhotra V. Non-autophagic roles of autophagy-related proteins. *EMBO Rep* (2013) 14(2):143–51. doi: 10.1038/embor.2012.220
234. Ahlers LRH, Goodman AG. Nucleic Acid Sensing and Innate Immunity: Signaling Pathways Controlling Viral Pathogenesis and Autoimmunity. *Curr Clin Microbiol Rep* (2016) 3(3):132–41. doi: 10.1007/s40588-016-0043-5
235. Tian Y, Wang M-L, Zhao J. Crosstalk between Autophagy and Type I Interferon Responses in Innate Antiviral Immunity. *Viruses* (2019) 11 (2):132. doi: 10.3390/v11020132
236. Liang Q, Seo GJ, Choi YJ, Kwak M-J, Ge J, Rodgers MA, et al. Crosstalk between the cGAS DNA Sensor and Beclin-1 Autophagy Protein Shapes Innate Antimicrobial Immune Responses. *Cell Host Microbe* (2014) 15 (2):228–38. doi: 10.1016/j.chom.2014.01.009
237. Randow F, Münz C. Autophagy in the regulation of pathogen replication and adaptive immunity. *Trends Immunol* (2012) 33(10):475–87. doi: 10.1016/j.it.2012.06.003
238. Van Kaer L, Parekh VV, Postoak JL, Wu L. Role of autophagy in MHC class I-restricted antigen presentation. *Mol Immunol* (2019) 113(October):2–5. doi: 10.1016/j.molimm.2017.10.021
239. Mintern JD, Macri C, Villadangos JA. Modulation of antigen presentation by intracellular trafficking. *Curr Opin Immunol* (2015) 34(Mhc I):16–21. doi: 10.1016/j.coi.2014.12.006
240. Joffre OP, Segura E, Savina A, Amigorena S. Cross-presentation by dendritic cells. *Nat Rev Immunol* (2012) 12(8):557–69. doi: 10.1038/nri3254
241. Noubade R, Majri-Morrison S, Tarbell KV. Beyond cDC1: Emerging Roles of DC Crosstalk in Cancer Immunity. *Front Immunol* (2019) 10:1014(MAY). doi: 10.3389/fimmu.2019.01014
242. Merzougui N, Kratzer R, Saveanu L, van Endert P. A proteasome-dependent, TAP-independent pathway for cross-presentation of phagocytosed antigen. *EMBO Rep* (2011) 12(12):1257–64. doi: 10.1038/embor.2011.203
243. Uhl M, Kepp O, Jusforgues-Saklani H, Vicencio J-M, Kroemer G, Albert ML. Autophagy within the antigen donor cell facilitates efficient antigen cross-priming of virus-specific CD8+ T cells. *Cell Death Differ* (2009) 16(7):991–1005. doi: 10.1038/cdd.2009.8
244. Münz C. Autophagy Beyond Intracellular MHC Class II Antigen Presentation. *Trends Immunol* (2016) 37(11):755–63. doi: 10.1016/j.it.2016.08.017
245. Schelhaas M. Viruses and cancer: molecular relations and perspectives. *Biol Chem* (2017) 398(8):815–6. doi: 10.1515/hsz-2017-0176
246. Kvansakul M, Hinds MG. Structural biology of the Bcl-2 family and its mimicry by viral proteins. *Cell Death Dis* (2013) 4(11):e909–9. doi: 10.1038/cddis.2013.436
247. Wimmer P, Schreiner S. Viral mimicry to usurp ubiquitin and sumo host pathways. *Viruses* (2015) 7(9):4854–77. doi: 10.3390/v7092849
248. Wong HH, Sanyal S. Manipulation of autophagy by (+) RNA viruses. *Semin Cell Dev Biol* (2020) 101(March):3–11. doi: 10.1016/j.semcdb.2019.07.013
249. Bird SW, Maynard ND, Covert MW, Kirkegaard K. Nonlytic viral spread enhanced by autophagy components. *Proc Natl Acad Sci* (2014) 111 (36):13081–6. doi: 10.1073/pnas.1401437111
250. Jackson WT, Giddings TH, Taylor MP, Mulinyawe S, Rabinovitch M, Kopito RR, et al. Subversion of Cellular Autophagosomal Machinery by RNA Viruses. Sugden B, ed. *PloS Biol* (2005) 3(5):e156. doi: 10.1371/journal.pbio.0030156
251. Alirezaei M, Flynn CT, Wood MR, Harkins S, Whitton JL. Coxsackievirus can exploit LC3 in both autophagy-dependent and -independent manners in vivo. *Autophagy* (2015) 11(8):1389–407. doi: 10.1080/15548627.2015.1063769
252. Mohamud Y, Shi J, Qu J, Poon T, Xue YC, Deng H, et al. Enteroviral Infection Inhibits Autophagic Flux via Disruption of the SNARE Complex to Enhance Viral Replication. *Cell Rep* (2018) 22(12):3292–303. doi: 10.1016/j.celrep.2018.02.090
253. Yoon SY, Ha YE, Choi JE, Ahn J, Leen H, Kweon H-S, et al. Coxsackievirus B4 Uses Autophagy for Replication after Calpain Activation in Rat Primary Neurons. *J Virol* (2008) 82(23):11976–8. doi: 10.1128/JVI.01028-08
254. Lee Y-R, Wang P-S, Wang J-R, Liu H-S. Enterovirus 71-induced autophagy increases viral replication and pathogenesis in a suckling mouse model. *J BioMed Sci* (2014) 21(1):80. doi: 10.1186/s12929-014-0080-4
255. Klein KA, Jackson WT. Human Rhinovirus 2 Induces the Autophagic Pathway and Replicates More Efficiently in Autophagic Cells. *J Virol* (2011) 85(18):9651–4. doi: 10.1128/JVI.00316-11
256. O'Donnell V, Pacheco JM, LaRocco M, Burrage T, Jackson W, Rodríguez LL, et al. Foot-and-mouth disease virus utilizes an autophagic pathway during viral replication. *Virology* (2011) 410(1):142–50. doi: 10.1016/j.virol.2010.10.042
257. Hou L, Ge X, Xin L, Zhou L, Guo X, Yang H. Nonstructural proteins 2C and 3D are involved in autophagy as induced by the encephalomyocarditis virus. *Virol J* (2014) 11(1):156. doi: 10.1186/1743-422X-11-156
258. Heaton NS, Randall G. Dengue Virus-Induced Autophagy Regulates Lipid Metabolism. *Cell Host Microbe* (2010) 8(5):422–32. doi: 10.1016/j.chom.2010.10.006
259. Zhang J, Lan Y, Li MY, Lamers MM, Fusade-Boyer M, Klemm E, et al. Flaviviruses Exploit the Lipid Droplet Protein AUP1 to Trigger Lipophagy and Drive Virus Production. *Cell Host Microbe* (2018) 23(6):819–31.e5. doi: 10.1016/j.chom.2018.05.005
260. Offerdahl DK, Dorward DW, Hansen BT, Bloom ME. Cytoarchitecture of Zika virus infection in human neuroblastoma and Aedes albopictus cell lines. *Virology* (2017) 501(November 2016):54–62. doi: 10.1016/j.virol.2016.11.002
261. Cortese M, Goellner S, Acosta EG, Neufeldt CJ, Oleksiuk O, Lampe M, et al. Ultrastructural Characterization of Zika Virus Replication Factories. *Cell Rep* (2017) 18(9):2113–23. doi: 10.1016/j.celrep.2017.02.014
262. de Haan CAM, Molinari M, Reggiori F. Autophagy-independent LC3 function in vesicular traffic. *Autophagy* (2010) 6(7):994–6. doi: 10.4161/auto.6.7.13309

263. Sun Y, Yu S, Ding N, Meng C, Meng S, Zhang S, et al. Autophagy Benefits the Replication of Newcastle Disease Virus in Chicken Cells and Tissues. *J Virol* (2014) 88(1):525–37. doi: 10.1128/JVI.01849-13
264. Prentice E, Jerome WG, Yoshimori T, Mizushima N, Denison MR. Coronavirus Replication Complex Formation Utilizes Components of Cellular Autophagy. *J Biol Chem* (2004) 279(11):10136–41. doi: 10.1074/jbc.M306124200
265. Krejbich-Trotot P, Gay B, Li-Pat-Yuen G, Hoarau J-J, Jaffar-Bandjee M-C, Briant L, et al. Chikungunya triggers an autophagic process which promotes viral replication. *Virol J* (2011) 8(1):432. doi: 10.1186/1743-422X-8-432
266. Sharma M, Bhattacharyya S, Sharma KB, Chauhan S, Asthana S, Abdin MZ, et al. Japanese encephalitis virus activates autophagy through XBP1 and ATF6 ER stress sensors in neuronal cells. *J Gen Virol* (2017) 98(5):1027–39. doi: 10.1099/jgv.0.000792
267. Dales S, Eggers HJ, Tamm I, Palade GE. Electron microscopic study of the formation of poliovirus. *Virology* (1965) 26(3):379–89. doi: 10.1016/0042-6822(65)90001-2
268. Jackson WT. Poliovirus-induced changes in cellular membranes throughout infection. *Curr Opin Virol* (2014) 9:67–73. doi: 10.1016/j.coviro.2014.09.007
269. Corona Velazquez A, Corona AK, Klein KA, Jackson WT. Poliovirus induces autophagic signaling independent of the ULK1 complex. *Autophagy* (2018) 14(7):1201–13. doi: 10.1080/15548627.2018.1458805
270. Wang H, Li K, Ma L, Wu S, Hu J, Yan H, et al. Berberine inhibits enterovirus 71 replication by downregulating the MEK/ERK signaling pathway and autophagy. *Virol J* (2017) 14(1):2. doi: 10.1186/s12985-016-0674-4
271. Rozières A, Viret C, Faure M. Autophagy in Measles Virus Infection. *Viruses* (2017) 9(12):359. doi: 10.3390/v9120359
272. Chiramel A, Brady N, Bartenschlager R. Divergent Roles of Autophagy in Virus Infection. *Cells* (2013) 2(1):83–104. doi: 10.3390/cells2010083
273. Joubert P-E, Meiffren G, Grégoire IP, Guillemette P, Clémence R, Flacher M, et al. Autophagy Induction by the Pathogen Receptor CD46. *Cell Host Microbe* (2009) 6(4):354–66. doi: 10.1016/j.chom.2009.09.006
274. Richetta C, Grégoire IP, Verlhac P, Azocar O, Baguet J, Flacher M, et al. Sustained Autophagy Contributes to Measles Virus Infectivity. Deretic V, ed. *PloS Pathog* (2013) 9(9):e1003599. doi: 10.1371/journal.ppat.1003599
275. Cordero R. Patogénesis del VIH / SIDA. *Rev Clinica la Esc Med* (2017) 7 (5):28–46.
276. Moir S, Fauci AS. B-cell responses to HIV infection. *Immunol Rev* (2017) 275 (1):33–48. doi: 10.1111/imr.12502
277. Yarchoan R, Uldrick TS. HIV-Associated Cancers and Related Diseases. Longo DL, ed. *N Engl J Med* (2018) 378(11):1029–41. doi: 10.1056/NEJMra1615896
278. Wood NH, Feller L. The malignant potential of HIV-associated Kaposi sarcoma. *Cancer Cell Int* (2008) 8(1):14. doi: 10.1186/1475-2867-8-14
279. Lu D-Y, Wu H-Y, Yarla NS, Xu B, Ding J, Lu T-R. HAART in HIV/AIDS Treatments: Future Trends. *Infect Disord Drug Targets* (2018) 18(1):15–22. doi: 10.2174/1871526517666170505122800
280. Tang S-W, Ducroux A, Jeang K-T, Neuveut C. Impact of cellular autophagy on viruses: Insights from hepatitis B virus and human retroviruses. *J BioMed Sci* (2012) 19(1):92. doi: 10.1186/1423-0127-19-92
281. Castro-Gonzalez S, Shi Y, Colomer-Lluch M, Song Y, Mowery K, Almodovar S, et al. HIV-1 Nef counteracts autophagy restriction by enhancing the association between BECN1 and its inhibitor BCL2 in a PRKN-dependent manner. *Autophagy* (2020) 00(00):1–25. doi: 10.1080/15548627.2020.1725401
282. Jia P. Autophagy and virus infection. *Infect Int* (2018) 6(4):124–8. doi: 10.2478/ii-2018-0001
283. Wang X, Gao Y, Tan J, Devadas K, Ragupathy V, Takeda K, et al. HIV-1 and HIV-2 infections induce autophagy in Jurkat and CD4+ T cells. *Cell Signal* (2012) 24(7):1414–9. doi: 10.1016/j.cellsig.2012.02.016
284. Neher RA, Dyrdak R, Druelle V, Hodcroft EB, Albert J. Potential impact of seasonal forcing on a SARS-CoV-2 pandemic. *Swiss Med Wkly* (2020) 150 (March):w20224. doi: 10.4414/smw.2020.20224
285. Raoult D, Zumla A, Locatelli F, Ippolito G, Kroemer G. Coronavirus infections: Epidemiological, clinical and immunological features and hypotheses. *Cell Stress* (2020) 4(4):66–75. doi: 10.15698/cst2020.04.216
286. Carmona-Gutierrez D, Bauer MA, Zimmermann A, Kainz K, Hofer SJ, Kroemer G, et al. Digesting the crisis: autophagy and coronaviruses. *Microb Cell* (2020) 2020:7(5):119–128. doi: 10.15698/mic2020.05.715
287. Petrova VN, Russell CA. The evolution of seasonal influenza viruses. *Nat Rev Microbiol* (2018) 16(1):47–60. doi: 10.1038/nrmicro.2017.118
288. Paulino J, Pang X, Hung I, Zhou H-X, Cross TA. Influenza A M2 Channel Clustering at High Protein/Lipid Ratios: Viral Budding Implications. *Biophys J* (2019) 116(6):1075–84. doi: 10.1016/j.bpj.2019.01.042
289. Beale R, Wise H, Stuart A, Ravenhill BJ, Digard P, Randow F. A LC3-Interacting Motif in the Influenza A Virus M2 Protein Is Required to Subvert Autophagy and Maintain Virion Stability. *Cell Host Microbe* (2014) 15 (2):239–47. doi: 10.1016/j.chom.2014.01.006
290. Joubert P-E, Werneke SW, de la Calle C, Florence G-B, Alessandra G, Lucie P, et al. Chikungunya virus–induced autophagy delays caspase-dependent cell death. *J Exp Med* (2012) 209(5):1029–47. doi: 10.1084/jem.20110996
291. Jordan TX, Randall G. Manipulation or capitulation: virus interactions with autophagy. *Microbes Infect* (2012) 14(2):126–39. doi: 10.1016/j.micinf.2011.09.007
292. Zompi S, Harris E. Animal Models of Dengue Virus Infection. *Viruses* (2012) 4(1):62–82. doi: 10.3390/v4010062
293. Heaton NS, Randall G. Dengue Virus and Autophagy. *Viruses* (2011) 3 (8):1332–41. doi: 10.3390/v3081332
294. Musso D, Ko AI, Baud D. Zika Virus Infection — After the Pandemic. Longo DL, ed. *N Engl J Med* (2019) 381(15):1444–57. doi: 10.1056/NEJMra1808246
295. Chiramel AI, Best SM. Role of autophagy in Zika virus infection and pathogenesis. *Virus Res* (2018) 254:34–40. doi: 10.1016/j.virusres.2017.09.006
296. Fishbein A, Hammock BD, Serhan CN, Panigrahy D. Carcinogenesis: Failure of resolution of inflammation? *Pharmacol Ther* (2020) 61(18):107670. doi: 10.1016/j.pharmthera.2020.107670
297. Kakavandi E, Shahbahrami R, Goudarzi H, Eslami G, Faghihloo E. Anoikis resistance and oncoviruses. *J Cell Biochem* (2018) 119(3):2484–91. doi: 10.1002/jcb.26363
298. Tornesello ML, Annunziata C, Tornesello AL, Buonaguro L, Buonaguro FM. Human Oncoviruses and p53 Tumor Suppressor Pathway Deregulation at the Origin of Human Cancers. *Cancers (Basel)* (2018) 10(7):213. doi: 10.3390/cancers10070213
299. Mesri EA, Feitelson MA, Munger K. Human Viral Oncogenesis: A Cancer Hallmarks Analysis. *Cell Host Microbe* (2014) 15(3):266–82. doi: 10.1016/j.chom.2014.02.011
300. Mui U, Haley C, Tyring S. Viral Oncology: Molecular Biology and Pathogenesis. *J Clin Med* (2017) 6(12):111. doi: 10.3390/jcm6120111
301. Krump NA, You J. Molecular mechanisms of viral oncogenesis in humans. *Nat Rev Microbiol* (2018) 16(11):684–98. doi: 10.1038/s41579-018-0064-6
302. Tornesello ML, Buonaguro L, Buonaguro FM. An overview of new biomolecular pathways in pathogen-related cancers. *Future Oncol* (2015) 11(11):1625–39. doi: 10.2217/fon.15.87
303. Akram N, Imran M, Noreen M, Ahmed F, Atif M, Fatima Z, et al. Oncogenic Role of Tumor Viruses in Humans. *Viral Immunol* (2017) 30(1):20–7. doi: 10.1089/vim.2016.0109
304. Jackson WT. Viruses and the autophagy pathway. *Virology* (2015) 479-480:450–6. doi: 10.1016/j.virol.2015.03.042
305. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. *Nature* (2011) 469(7330):323–35. doi: 10.1038/nature09782
306. Haley CT, Mui UN, Vangipuram R, Rady PL, Tyring SK. Human oncoviruses: Mucocutaneous manifestations, pathogenesis, therapeutics, and prevention. *J Am Acad Dermatol* (2019) 81(1):1–21. doi: 10.1016/j.jaad.2018.09.062
307. Ringehan M, McKeating JA, Protzer U. Viral hepatitis and liver cancer. *Philos Trans R Soc B Biol Sci* (2017) 372(1732):20160274. doi: 10.1098/rstb.2016.0274
308. Cao J, Li D. Searching for human oncoviruses: Histories, challenges, and opportunities. *J Cell Biochem* (2018) 119(6):4897–906. doi: 10.1002/jcb.26717
309. Herbein G. The Human Cytomegalovirus, from Oncomodulation to Oncogenesis. *Viruses* (2018) 10(8):408. doi: 10.3390/v10080408
310. Yamagishi M, Fujikawa D, Watanabe T, Uchimaru K. HTLV-1-Mediated Epigenetic Pathway to Adult T-Cell Leukemia–Lymphoma. *Front Microbiol* (2018) 9:1686(JUL). doi: 10.3389/fmicb.2018.01686
311. Vescovo T, Pagni B, Piacentini M, Fimia GM, Antonioli M. Regulation of Autophagy in Cells Infected With Oncogenic Human Viruses and Its Impact

on Cancer Development. *Front Cell Dev Biol* (2020) 8:47(February). doi: 10.3389/fcell.2020.00047
312. McElwee M, Vijayakrishnan S, Rixon F, Bhella D. Structure of the herpes simplex virus portal-vertex. Sugden B, ed. *PloS Biol* (2018) 16(6):e2006191. doi: 10.1371/journal.pbio.2006191
313. Agelidis AM, Shukla D. Cell entry mechanisms of HSV: what we have learned in recent years. *Future Virol* (2015) 10(10):1145–54. doi: 10.2217/fvl.15.85
314. Jha H, Banerjee S, Robertson E. The Role of Gammaherpesviruses in Cancer Pathogenesis. *Pathogens* (2016) 5(1):18. doi: 10.3390/pathogens5010018
315. Chang Y, Moore PS, Weiss RA. Human oncogenic viruses: nature and discovery. *Philos Trans R Soc B Biol Sci* (2017) 372(1732):20160264. doi: 10.1098/rstb.2016.0264
316. Cirone M. EBV and KSHV Infection Dysregulates Autophagy to Optimize Viral Replication, Prevent Immune Recognition and Promote Tumorigenesis. *Viruses* (2018) 10(11):599. doi: 10.3390/v10110599
317. Chang Y, Cesarman E, Pessin M, Lee F, Culpepper J, Knowles D, et al. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science (80-)* (1994) 266(5192):1865–9. doi: 10.1126/science.7997879
318. Calabrò ML, Sarid R. Human Herpesvirus 8 And Lymphoproliferative Diseases. *Mediterr J Hematol Infect Dis* (2018) 10(1):e2018061. doi: 10.4084/mjhid.2018.061
319. De Paoli P, Carbone A. Kaposi's Sarcoma Herpesvirus: twenty years after its discovery. *Eur Rev Med Pharmacol Sci* (2016) 20(7):1288–94.
320. Wakeman BS, Izumiya Y, Speck SH. Identification of Novel Kaposi's Sarcoma-Associated Herpesvirus Orf50 Transcripts: Discovery of New RTA Isoforms with Variable Transactivation Potential. Jung JU, ed. *J Virol* (2017) 91(1):1–23. doi: 10.1128/JVI.01434-16
321. Cesarman E, Damania B, Krown SE, Martin J, Bower M, Whitby D. Kaposi sarcoma. *Nat Rev Dis Primers* (2019) 5(1):9. doi: 10.1038/s41572-019-0060-9
322. Veettil M, Bandyopadhyay C, Dutta D, Chandran B. Interaction of KSHV with Host Cell Surface Receptors and Cell Entry. *Viruses* (2014) 6(10):4024–46. doi: 10.3390/v6104024
323. Giffin L, Damania B. Chapter Two-KSHV: Pathways to Tumorigenesis and Persisten Infection. *Advances in Virus Research*, *1st ed*, vol. 88. . Elsevier Inc (2014). p. 111–59. doi: 10.1016/B978-0-12-800098-4.00002-7
324. Rosario SA, Santiago GE, Mesri EA, Verdun RE. Kaposi's Sarcoma-Associated Herpesvirus-Encoded Viral IL-6 (vIL-6) Enhances Immunoglobulin Class-Switch Recombination. *Front Microbiol* (2018) 9:3119(December). doi: 10.3389/fmicb.2018.03119
325. Choi Y, Bowman JW, Jung JU. Autophagy during viral infection — a double-edged sword. *Nat Rev Microbiol* (2018) 16(6):341–54. doi: 10.1038/s41579-018-0003-6
326. Ueda K. KSHV Genome Replication and Maintenance in Latency. *Adv Exp Med Biol* (2018) 1045:299–320. doi: 10.1007/978-981-10-7230-7_14
327. Wang L. The Kaposi's Sarcoma-Associated Herpesvirus (KSHV/HHV-8) K1 Protein Induces Expression of Angiogenic and Invasion Factors. *Cancer Res* (2004) 64(8):2774–81. doi: 10.1158/0008-5472.CAN-03-3653
328. Leidal AM, Cyr DP, Hill RJ, Lee PWK, McCormick C. Subversion of Autophagy by Kaposi's Sarcoma-Associated Herpesvirus Impairs Oncogene-Induced Senescence. *Cell Host Microbe* (2012) 11(2):167–80. doi: 10.1016/j.chom.2012.01.005
329. Anders PM, Zhang Z, Bhende PM, Giffin L, Damania B. The KSHV K1 Protein Modulates AMPK Function to Enhance Cell Survival. Schulz TF, ed. *PloS Pathog* (2016) 12(11):e1005985. doi: 10.1371/journal.ppat.1005985
330. Yin H, Shao S, Jiang X, Xie P, Sun W, Yu T. Interactions between Autophagy and DNA Viruses. *Viruses* (2019) 11(9):776. doi: 10.3390/v11090776
331. He M, Zhang W, Bakken T, Schutten M, Toth Z, Jung JU, et al. Cancer Angiogenesis Induced by Kaposi Sarcoma-Associated Herpesvirus Is Mediated by EZH2. *Cancer Res* (2012) 72(14):3582–92. doi: 10.1158/0008-5472.CAN-11-2876
332. Granato M, Santarelli R, Filardi M, Gonnrlls R, Farina A, Torrisi MR, et al. The activation of KSHV lytic cycle blocks autophagy in PEL cells. *Autophagy* (2015) 11(11):1978–86. doi: 10.1080/15548627.2015.1091911
333. Santarelli R, Gonnella R, Di Giovenale G, Cuomo L, Capobianchi A, Granato M, et al. STAT3 activation by KSHV correlates with IL-10, IL-6 and IL-23 release and an autophagic block in dendritic cells. *Sci Rep* (2015) 4(1):4241. doi: 10.1038/srep04241
334. Yan L, Majerciak V, Zheng Z-M, Lan K. Towards Better Understanding of KSHV Life Cycle: from Transcription and Posttranscriptional Regulations to Pathogenesis. *Virol Sin* (2019) 34(2):135–61. doi: 10.1007/s12250-019-00114-3
335. Broussard G, Damania B. Regulation of KSHV Latency and Lytic Reactivation. *Viruses* (2020) 12(9):1034. doi: 10.3390/v12091034
336. De Leo A, Chen H-S, Hu C-CA, Lieberman PM. Deregulation of KSHV latency conformation by ER-stress and caspase-dependent RAD21-cleavage. Flemington EK, ed. *PloS Pathog* (2017) 13(8):e1006596. doi: 10.1371/journal.ppat.1006596
337. Guito J, Lukac D. KSHV Reactivation and Novel Implications of Protein Isomerization on Lytic Switch Control. *Viruses* (2015) 7(1):72–109. doi: 10.3390/v7010072
338. Pringle ES, Robinson C-A, McCormick C. Kaposi's Sarcoma-Associated Herpesvirus Lytic Replication Interferes with mTORC1 Regulation of Autophagy and Viral Protein Synthesis. Jung JU, ed. *J Virol* (2019) 93 (21):1–22. doi: 10.1128/JVI.00854-19
339. Wen H-J, Yang Z, Zhou Y, Wood C. Enhancement of Autophagy during Lytic Replication by the Kaposi's Sarcoma-Associated Herpesvirus Replication and Transcription Activator. *J Virol* (2010) 84(15):7448–58. doi: 10.1128/JVI.00024-10
340. Mariggiò G, Koch S, Zhang G, Weidner-Glunde M, Rückert J, Kati S, et al. Kaposi Sarcoma Herpesvirus (KSHV) Latency-Associated Nuclear Antigen (LANA) recruits components of the MRN (Mre11-Rad50-NBS1) repair complex to modulate an innate immune signaling pathway and viral latency. Robertson ES, ed. *PloS Pathog* (2017) 13(4):e1006335. doi: 10.1371/journal.ppat.1006335
341. Gilardini Montani MS, Falcinelli L, Santarelli R, Romeo MA, Granato M, Faggioni A, et al. Kaposi Sarcoma Herpes Virus (KSHV) infection inhibits macrophage formation and survival by counteracting Macrophage Colony-Stimulating Factor (M-CSF)-induced increase of Reactive Oxygen Species (ROS), c-Jun N-terminal kinase (JNK) phosphorylation and auto. *Int J Biochem Cell Biol* (2019) 114(June):105560. doi: 10.1016/j.biocel.2019.06.008
342. Cai Q, Chen K, Young KH. Epstein–Barr virus-positive T/NK-cell lymphoproliferative disorders. *Exp Mol Med* (2015) 47(1):e133–3. doi: 10.1038/emm.2014.105
343. Shinozaku-Ushiku A, Kunita A, Fukuyama M. Update on Epstein-Barr virus and gastric cancer (Review). *Int J Oncol* (2015) 46(4):1421–34. doi: 10.3892/ijo.2015.2856
344. Stanfield BA, Luftig MA. Recent advances in understanding Epstein-Barr virus. *F1000Research* (2017) 6(0):386. doi: 10.12688/f1000research.10591.1
345. Pujals A, Favre L, Pioche-Durieu C, Robert A, Meurice G, Le Gentil M, et al. Constitutive autophagy contributes to resistance to TP53-mediated apoptosis in Epstein-Barr virus-positive latency III B-cell lymphoproliferations. *Autophagy* (2015) 11(12):2275–87. doi: 10.1080/15548627.2015.1115939
346. Farrell PJ. Epstein–Barr Virus and Cancer. *Annu Rev Pathol Mech Dis* (2019) 14(1):29–53. doi: 10.1146/annurev-pathmechdis-012418-013023
347. Ascherio A, Munger KL. Epstein–Barr Virus Infection and Multiple Sclerosis: A Review. *J Neuroimmune Pharmacol* (2010) 5(3):271–7. doi: 10.1007/s11481-010-9201-3
348. Chen J. Roles of the PI3K/Akt pathway in Epstein-Barr virus-induced cancers and therapeutic implications. *World J Virol* (2012) 1(6):154. doi: 10.5501/wjv.v1.i6.154
349. Fang W-L, Huang K-H, Lan Y-T, Lin C-H, Chang S-C, Chen M-H, et al. Mutations in PI3K/AKT pathway genes and amplifications of PI3KCA are associated with patterns of recurrence in gastric cancers. *Oncotarget* (2016) 7 (5):6201–20. doi: 10.18632/oncotarget.6641
350. Cen O, Longnecker R. Latent Membrane Protein 2 (LMP2). Epstein Barr Virus Volume 2. *Curr Top Microbiol Immunol* (2015) 391:151–80. doi: 10.1007/978-3-319-22834-1_5
351. Fish K, Sora RP, Schaller SJ, Longnecker R, Ikeda M. EBV latent membrane protein 2A orchestrates p27kip1 degradation via Cks1 to accelerate MYC-driven lymphoma in mice. *Blood* (2017) 130(23):2516–26. doi: 10.1182/blood-2017-07-796821
352. Smatti MK, Al-Sadeq DW, Ali NH, Pintus G, Abou-Saleh H, Nasrallah GK. Epstein–Barr Virus Epidemiology, Serology, and Genetic Variability of LMP-

1 Oncogene Among Healthy Population: An Update. *Front Oncol* (2018) 8:211(JUN):1–16. doi: 10.3389/fonc.2018.00211
353. Hurwitz SN, Cheerathodi MR, Nkosi D, York SB, Meckes DG. Tetraspanin CD63 Bridges Autophagic and Endosomal Processes To Regulate Exosomal Secretion and Intracellular Signaling of Epstein-Barr Virus LMP1. Longnecker RM, ed. *J Virol* (2017) 92(5):1–21. doi: 10.1128/JVI.01969-17
354. Bhattacharjee S, Bose P, Patel K, Roy SG, Gain C, Gowda H, et al. Transcriptional and epigenetic modulation of autophagy promotes EBV oncoprotein EBNA3C induced B-cell survival. *Cell Death Dis* (2018) 9 (6):605. doi: 10.1038/s41419-018-0668-9
355. De Leo A, Colavita F, Ciccosanti F, Fimia GM, Lieberman PM, Mattia E. Inhibition of autophagy in EBV-positive Burkitt's lymphoma cells enhances EBV lytic genes expression and replication. *Cell Death Dis* (2015) 6(9): e1876–6. doi: 10.1038/cddis.2015.156
356. Nowag H, Guhl B, Thriene K, Romao S, Ziegler U, Dengjel J, et al. Macroautophagy Proteins Assist Epstein Barr Virus Production and Get Incorporated Into the Virus Particles. *EBioMedicine* (2014) 1(2-3):116–25. doi: 10.1016/j.ebiom.2014.11.007
357. Granato M, Santarelli R, Farina A, Gonnela R, Lotti LV, Faggioni A, et al. Epstein-Barr Virus Blocks the Autophagic Flux and Appropriates the Autophagic Machinery To Enhance Viral Replication. *J Virol* (2014) 88 (21):12715–26. doi: 10.1128/JVI.02199-14
358. Hung C-H, Chen L-W, Wang W-H, Chang P-J, Chiu Y-F, Hung C-C, et al. Regulation of Autophagic Activation by Rta of Epstein-Barr Virus via the Extracellular Signal-Regulated Kinase Pathway. *J Virol* (2014) 88(20):12133–45. doi: 10.1128/JVI.02033-14
359. Khanim F, Mackett M, Young LS, Dawson J, Meseda CA, Dawson C. BHRF1, a viral homologue of the Bcl-2 oncogene, is conserved at both the sequence and functional level in different Epstein-Barr virus isolates. *J Gen Virol* (1997) 78(11):2987–99. doi: 10.1099/0022-1317-78-11-2987
360. Marshall WL, Yim C, Gustafson E, Graf T, Sage DR, Hanify K, et al. Epstein-Barr Virus Encodes a Novel Homolog of the bcl-2 Oncogene That Inhibits Apoptosis and Associates with Bax and Bak. *J Virol* (1999) 73(6):5181–5. doi: 10.1128/JVI.73.6.5181-5185.1999
361. Altmann M, Hammerschmidt W. Epstein-Barr Virus Provides a New Paradigm: A Requirement for the Immediate Inhibition of Apoptosis. Sugden B, ed. *PloS Biol* (2005) 3(12):e404. doi: 10.1371/journal.pbio.0030404
362. Henderson S, Huen D, Rowe M, Dawson C, Johnson G, Rickinson A. Epstein-Barr virus-coded BHRF1 protein, a viral homologue of Bcl-2, protects human B cells from programmed cell death. *Proc Natl Acad Sci U S A* (1993) 90(18):8479–83. doi: 10.1073/pnas.90.18.8479
363. Bellows DS, Howell M, Pearson C, Hazlewood SA, Hardwick JM. Epstein-Barr Virus BALF1 Is a BCL-2-Like Antagonist of the Herpesvirus Antiapoptotic BCL-2 Proteins. *J Virol* (2002) 76(5):2469–79. doi: 10.1128/jvi.76.5.2469-2479.2002
364. Shao Z, Borde C, Quignon F, Escargueil A, Maréchal V. Epstein–Barr Virus BALF0 and BALF1 Modulate Autophagy. *Viruses* (2019) 11(12):1099. doi: 10.3390/v11121099
365. Plotkin SA, Boppana SB. Vaccination against the human cytomegalovirus. *Vaccine* (2019) 37(50):7437–42. doi: 10.1016/j.vaccine.2018.02.089
366. Lussignol M, Esclatine A. Cytomegalovirus and Autophagy. *Immunology* (2018) 1:9–21. doi: 10.1016/B978-0-12-809819-6.00002-2
367. Joseph GP, McDermott R, Baryshnikova MA, Cobbs CS, Ulasov IV. Cytomegalovirus as an oncomodulatory agent in the progression of glioma. *Cancer Lett* (2017) 384:79–85. doi: 10.1016/j.canlet.2016.10.022
368. Chaumorcel M, Lussignol M, Mouna L, Cavignac Y, Fahie K, Cotte-Laffitte J, et al. The Human Cytomegalovirus Protein TRS1 Inhibits Autophagy via Its Interaction with Beclin 1. *J Virol* (2012) 86(5):2571–84. doi: 10.1128/JVI.05746-11
369. Mouna L, Hernandez E, Bonte D, Brost R, Amazit L, Delgui LR, et al. Analysis of the role of autophagy inhibition by two complementary human cytomegalovirus BECN1/Beclin 1-binding proteins. *Autophagy* (2016) 12 (2):327–42. doi: 10.1080/15548627.2015.1125071
370. Mlera L, Moy M, Maness K, Tran LN, Goodrum FD. The Role of the Human Cytomegalovirus UL133-UL138 Gene Locus in Latency and Reactivation. *Viruses* (2020) 12(7):714. doi: 10.3390/v12070714
371. Tey SK, Khanna R. Autophagy mediates transporter associated with antigen processing- independent presentation of viral epitopes through MHC class I pathway. *Blood* (2012) 120(5):994–1004. doi: 10.1182/blood-2012-01-402404
372. Loi M, Müller A, Steinbach K, Niven J, Barreira da Silva R, Paul P, et al. Macroautophagy Proteins Control MHC Class I Levels on Dendritic Cells and Shape Anti-viral CD8 + T Cell Responses. *Cell Rep* (2016) 15(5):1076–87. doi: 10.1016/j.celrep.2016.04.002
373. Reddehase MJ, Lemmermann NAW. Cellular reservoirs of latent cytomegaloviruses. *Med Microbiol Immunol* (2019) 208(3-4):391–403. doi: 10.1007/s00430-019-00592-y
374. Møller R, Schwarz TM, Noriega VM, Panis M, Sachs D, Tortorella D, et al. miRNA-mediated targeting of human cytomegalovirus reveals biological host and viral targets of IE2. *Proc Natl Acad Sci* (2018) 115(5):1069–74. doi: 10.1073/pnas.1719036115
375. Li M, Ball CB, Collins G, Hu Q, Luse DS, Price DH, et al. Human cytomegalovirus IE2 drives transcription initiation from a select subset of late infection viral promoters by host RNA polymerase II. Glaunsinger BA, ed. *PloS Pathog* (2020) 16(4):e1008402. doi: 10.1371/journal.ppat.1008402
376. Zhang X, Xi T, Zhang L, Bi Y, Huang Y, Lu Y, et al. The role of autophagy in human cytomegalovirus IE2 expression. *J Med Virol* 2020) 25:jmv.26357. doi: 10.1002/jmv.26357
377. Martin J, Maldonado J, Mueller J, Zhang W, Mansky L. Molecular Studies of HTLV-1 Replication: An Update. *Viruses* (2016) 8(2):31. doi: 10.3390/v8020031
378. Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci* (1980) 77(12):7415–9. doi: 10.1073/pnas.77.12.7415
379. Futsch N, Prates G, Mahieux R, Casseb J, Dutartre H. Cytokine Networks Dysregulation during HTLV-1 Infection and Associated Diseases. *Viruses* (2018) 10(12):691. doi: 10.3390/v10120691
380. Schierhout G, McGregor S, Gessain A, Einsiedel L, Martinello M, Kaldor J. Association between HTLV-1 infection and adverse health outcomes: a systematic review and meta-analysis of epidemiological studies. *Lancet Infect Dis* (2020) 20(1):133–43. doi: 10.1016/S1473-3099(19)30402-5
381. Gessain A, Cassar O. Epidemiological Aspects and World Distribution of HTLV-1 Infection. *Front Microbiol* (2012) 3:388(NOV). doi: 10.3389/fmicb.2012.00388
382. Tang S-W, Chen C-Y, Klase Z, Zane L, Jeang K-T. The Cellular Autophagy Pathway Modulates Human T-Cell Leukemia Virus Type 1 Replication. *J Virol* (2013) 87(3):1699–707. doi: 10.1128/JVI.02147-12
383. Chen L, Liu D, Zhang Y, Zhang H, Cheng H. The autophagy molecule Beclin 1 maintains persistent activity of NF-κB and Stat3 in HTLV-1-transformed T lymphocytes. *Biochem Biophys Res Commun* (2015) 465(4):739–45. doi: 10.1016/j.bbrc.2015.08.070
384. Nakahata S, Syahrul C, Nakatake A, Sakamoto K, Yoshihama M, Nishikata I, et al. Clinical significance of soluble CADM1 as a novel marker for adult T-cell leukemia/lymphoma. *Haematologica* (2020) 106(2):532–42. doi: 10.3324/haematol.2019.234096
385. Sarkar B, Nishikata I, Nakahata S, Ichikawa T, Shiraga T, Saha HR, et al. Degradation of p47 by autophagy contributes to CADM1 overexpression in ATLL cells through the activation of NF-κB. *Sci Rep* (2019) 9(1):3491. doi: 10.1038/s41598-019-39424-7
386. Mukai R, Ohshima T. HTLV-1 HBZ positively regulates the mTOR signaling pathway via inhibition of GADD34 activity in the cytoplasm. *Oncogene* (2014) 33(18):2317–28. doi: 10.1038/onc.2013.181
387. Baratella M, Forlani G, Accolla RS. HTLV-1 HBZ Viral Protein: A Key Player in HTLV-1 Mediated Diseases. *Front Microbiol* (2017) 8:2615(DEC). doi: 10.3389/fmicb.2017.02615
388. Ren T, Takahashi Y, Liu X, Loughran TP, Sun S-C, Wang H-G, et al. HTLV-1 Tax deregulates autophagy by recruiting autophagic molecules into lipid raft microdomains. *Oncogene* (2015) 34(3):334–45. doi: 10.1038/onc.2013.552
389. Manns MP, Buti M, Gane E, Pawlotsky J-M, Razavi H, Terrault N, et al. Hepatitis C virus infection. *Nat Rev Dis Primers* (2017) 3(1):17006. doi: 10.1038/nrdp.2017.6
390. Balakrishnan M, Glover MT, Kanwal F. Hepatitis C and Risk of Nonhepatic Malignancies. *Clin Liver Dis* (2017) 21(3):543–54. doi: 10.1016/j.cld.2017.03.009
391. Pol S, Vallet-Pichard A, Hermine O. Extrahepatic cancers and chronic HCV infection. *Nat Rev Gastroenterol Hepatol* (2018) 15(5):283–90. doi: 10.1038/nrgastro.2017.172

392. Paul D, Hoppe S, Saher G, Krijnse-Locker J, Bartenschlager R. Morphological and Biochemical Characterization of the Membranous Hepatitis C Virus Replication Compartment. *J Virol* (2013) 87(19):10612–27. doi: 10.1128/JVI.01370-13
393. Ferraris P, Beaumont E, Uzbekov R, Brand D, Gaillard J, Blanchard E, et al. Sequential biogenesis of host cell membrane rearrangements induced by hepatitis C virus infection. *Cell Mol Life Sci* (2013) 70(7):1297–306. doi: 10.1007/s00018-012-1213-0
394. Vescovo T, Refolo G, Romagnoli A, Ciccosanti F, Corazzari M, Alonzi T, et al. Autophagy in HCV Infection: Keeping Fat and Inflammation at Bay. *BioMed Res Int* (2014) 2014:1–10. doi: 10.1155/2014/265353
395. Fahmy AM, Labonté P. The autophagy elongation complex (ATG5-12/16L1) positively regulates HCV replication and is required for wild-type membranous web formation. *Sci Rep* (2017) 7(1):40351. doi: 10.1038/srep40351
396. Wang L, James Ou J. Hepatitis C virus and autophagy. *Biol Chem* (2015) 396 (11):1215–22. doi: 10.1515/hsz-2015-0172
397. Jassey A, Liu C-H, Changou C, Richardson C, Hsu H-Y, Lin L-T. Hepatitis C Virus Non-Structural Protein 5A (NS5A) Disrupts Mitochondrial Dynamics and Induces Mitophagy. *Cells* (2019) 8(4):290. doi: 10.3390/cells8040290
398. Kim S-J, Syed GH, Siddiqui A. Hepatitis C Virus Induces the Mitochondrial Translocation of Parkin and Subsequent Mitophagy. Ou JJ, ed. *PloS Pathog* (2013) 9(3):e1003285. doi: 10.1371/journal.ppat.1003285
399. Seto W-K, Lo Y-R, Pawlotsky J-M, Yuen M-F. Chronic hepatitis B virus infection. *Lancet* (2018) 392(10161):2313–24. doi: 10.1016/S0140-6736(18)31865-8
400. Herrscher C, Roingeard P, Blanchard E. Hepatitis B Virus Entry into Cells. *Cells* (2020) 9(6):1486. doi: 10.3390/cells9061486
401. Schinzari V, Barnaba V, Piconese S. Chronic hepatitis B virus and hepatitis C virus infections and cancer: synergy between viral and host factors. *Clin Microbiol Infect* (2015) 21(11):969–74. doi: 10.1016/j.cmi.2015.06.026
402. Seeger C, Mason WS. Molecular biology of hepatitis B virus infection. *Virology* (2015) 479-480:672–86. doi: 10.1016/j.virol.2015.02.031
403. Ringelhan M, Heikenwalder M, Protzer U. Direct Effects of Hepatitis B Virus-Encoded Proteins and Chronic Infection in Liver Cancer Development. *Dig Dis* (2013) 31(1):138–51. doi: 10.1159/000347209
404. Li J, Liu Y, Wang Z, Liu K, Wang Y, Liu J, et al. Subversion of Cellular Autophagy Machinery by Hepatitis B Virus for Viral Envelopment. *J Virol* (2011) 85(13):6319–33. doi: 10.1128/JVI.02627-10
405. Tang H, Da L, Mao Y, Li Y, Li D, Xu Z, et al. Hepatitis B virus X protein sensitizes cells to starvation-induced autophagy via up-regulation of beclin 1 expression. *Hepatology* (2009) 49(1):60–71. doi: 10.1002/hep.22581
406. Bouchard MJ, Schneider RJ. The Enigmatic X Gene of Hepatitis B Virus. *J Virol* (2004) 78(23):12725–34. doi: 10.1128/JVI.78.23.12725-12734.2004
407. Zhang H-T, Chen GG, Hu B-G, Zhang Z-Y, Yun J-P, He M-L, et al. Hepatitis B virus x protein induces autophagy via activating death-associated protein kinase. *J Viral Hepat* (2014) 21(9):642–9. doi: 10.1111/jvh.12191
408. Liu B, Fang M, Hu Y, Huang B, Li N, Chang C, et al. Hepatitis B virus X protein inhibits autophagic degradation by impairing lysosomal maturation. *Autophagy* (2014) 10(3):416–30. doi: 10.4161/auto.27286
409. Zhou T, Jin M, Ding Y, Zhang Y, Sun Y, Huang S, et al. Hepatitis B virus dampens autophagy maturation via negative regulation of Rab7 expression. *Biosci Trends* (2016) 10(4):244–50. doi: 10.5582/bst.2016.01049
410. Kim S-J, Khan M, Quan J, Till A, Subramani S, Siddiqui A. Hepatitis B Virus Disrupts Mitochondrial Dynamics: Induces Fission and Mitophagy to Attenuate Apoptosis. Luo G, ed. *PloS Pathog* (2013) 9(12):e1003722. doi: 10.1371/journal.ppat.1003722
411. Wang X, Lin Y, Kemper T, Chen J, Yuan Z, Liu S, et al. AMPK and Akt/mTOR signalling pathways participate in glucose-mediated regulation of hepatitis B virus replication and cellular autophagy. *Cell Microbiol* (2020) 22 (2):1–16. doi: 10.1111/cmi.13131
412. Chen L, Ming X, Li W, Bi M, Yan B, Wang X, et al. The microRNA-155 mediates hepatitis B virus replication by reinforcing SOCS1 signalling–induced autophagy. *Cell Biochem Funct* (2020) 38(4):436–42. doi: 10.1002/cbf.3488
413. Wu S-Y. Autophagy and microRNA in hepatitis B virus-related hepatocellular carcinoma. *World J Gastroenterol* (2016) 22(1):176. doi: 10.3748/wjg.v22.i1.176
414. Ballestar E, Li T. New insights into the epigenetics of inflammatory rheumatic diseases. *Nat Rev Rheumatol* (2017) 13(10):593–605. doi: 10.1038/nrrheum.2017.147
415. Liu D, Li P, Guo J, Li L-L, Guo B, Jiao H-H, et al. Exosomes derived from HBV–associated liver cancer promote chemoresistance by upregulating chaperone–mediated autophagy. *Oncol Lett* (2018) 17(1):323–31. doi: 10.3892/ol.2018.9584
416. Moens U. Human Polyomaviruses and Papillomaviruses. *Int J Mol Sci* (2018) 19(8):2360. doi: 10.3390/ijms19082360
417. Moens U, Rasheed K, Abdulsalam I, Sveinbjørnsson B. The Role of Merkel Cell Polyomavirus and Other Human Polyomaviruses in Emerging Hallmarks of Cancer. *Viruses* (2015) 7(4):1871–901. doi: 10.3390/v7041871
418. Liu W, MacDonald M, You J. Merkel cell polyomavirus infection and Merkel cell carcinoma. *Curr Opin Virol* (2016) 20:20–7. doi: 10.1016/j.coviro.2016.07.011
419. Grundhoff A, Fischer N. Merkel cell polyomavirus, a highly prevalent virus with tumorigenic potential. *Curr Opin Virol* (2015) 14:129–37. doi: 10.1016/j.coviro.2015.08.010
420. Wendzicki JA, Moore PS, Chang Y. Large T and small T antigens of Merkel cell polyomavirus. *Curr Opin Virol* (2015) 11:38–43. doi: 10.1016/j.coviro.2015.01.009
421. Borchert S, Czech-Sioli M, Neumann F, Schmidt C, Wimmer P, Dobner T, et al. High-Affinity Rb Binding, p53 Inhibition, Subcellular Localization, and Transformation by Wild-Type or Tumor-Derived Shortened Merkel Cell Polyomavirus Large T Antigens. *J Virol* (2014) 88(6):3144–60. doi: 10.1128/JVI.02916-13
422. Berrios C, Padi M, Keibler MA, Park DE, Molla V, Cheng J, et al. Merkel Cell Polyomavirus Small T Antigen Promotes Pro-Glycolytic Metabolic Perturbations Required for Transformation. Kalejta RF, ed. *PloS Pathog* (2016) 12(11):e1006020. doi: 10.1371/journal.ppat.1006020
423. Shuda M, Arora R, Kwun HJ, Feng H, Sarid R, Fernández-Figueras M-T, et al. Human Merkel cell polyomavirus infection I. MCV T antigen expression in Merkel cell carcinoma, lymphoid tissues and lymphoid tumors. *Int J Cancer* (2009) 125(6):1243–9. doi: 10.1002/ijc.24510
424. Verhaegen ME, Mangelberger D, Harms PW, Vozheiko TD, Weich JW, Wilbert DM, et al. Merkel Cell Polyomavirus Small T Antigen Is Oncogenic in Transgenic Mice. *J Invest Dermatol* (2015) 135(5):1415–24. doi: 10.1038/jid.2014.446
425. Kumar S, Xie H, Shi H, Gao J, Juhlin CC, Bjornhagen V, et al. Merkel cell polyomavirus oncoproteins induce microRNAs that suppress multiple autophagy genes. *Int J Cancer* (2020) 146(6):1652–66. doi: 10.1002/ijc.32503
426. Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S. Human papillomavirus and cervical cancer. *Lancet* (2007) 370(9590):890–907. doi: 10.1016/S0140-6736(07)61416-0
427. Berman TA, Schiller JT. Human papillomavirus in cervical cancer and oropharyngeal cancer: One cause, two diseases. *Cancer* (2017) 123 (12):2219–29. doi: 10.1002/cncr.30588
428. DiGiuseppe S, Bienkowska-Haba M, Sapp M. Human Papillomavirus Entry: Hiding in a Bubble. Tsai B, ed. *J Virol* (2016) 90(18):8032–5. doi: 10.1128/JVI.01065-16
429. Mattoscio D, Medda A, Chiocca S. Human Papilloma Virus and Autophagy. *Int J Mol Sci* (2018) 19(6):1775. doi: 10.3390/ijms19061775
430. Kim YC, Guan KL. MTOR: A pharmacologic target for autophagy regulation. *J Clin Invest* (2015) 125(1):25–32. doi: 10.1172/JCI73939
431. Papinski D, Kraft C. Regulation of Autophagy By Signaling Through the Atg1/ULK1 Complex. *J Mol Biol* (2016) 428(9):1725–41. doi: 10.1016/j.jmb.2016.03.030
432. Sapp M, Bienkowska-Haba M. Viral entry mechanisms: human papillomavirus and a long journey from extracellular matrix to the nucleus. *FEBS J* (2009) 276 (24):7206–16. doi: 10.1111/j.1742-4658.2009.07400.x
433. Pinidis P, Tsikouras P, Iatrakis G, Zervoudis S, Koukouli Z, Bothou A, et al. Human Papilloma Virus' Life Cycle and Carcinogenesis. *Maedica (Buchar)* (2016) 11(1):48–54.
434. Belleudi F, Nanni M, Raffa S, Torrisi MR. HPV16 E5 deregulates the autophagic process in human keratinocytes. *Oncotarget* (2015) 6(11):9370–86. doi: 10.18632/oncotarget.3326
435. Mattoscio D, Casadio C, Miccolo C, Maffini F, Raimondi A, Tacchetti C, et al. Autophagy regulates UBC9 levels during viral-mediated tumorigenesis. Raab-Traub N, ed. *PloS Pathog* (2017) 13(3):e1006262. doi: 10.1371/journal.ppat.1006262
436. Dayyani F, Etzel CJ, Liu M, Ho C-H, Lippman SM, Tsao AS. Meta-analysis of the impact of human papillomavirus (HPV) on cancer risk and overall

survival in head and neck squamous cell carcinomas (HNSCC). *Head Neck Oncol* (2010) 2(1):15. doi: 10.1186/1758-3284-2-15

437. Sannigrahi M, Singh V, Sharma R, Panda N, Khullar M. Role of autophagy in head and neck cancer and therapeutic resistance. *Oral Dis* (2015) 21(3):283–91. doi: 10.1111/odi.12254

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a shared affiliation, though no other collaboration, with the authors.

Copyright © 2021 Suares, Medina and Coso. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Oncology

REVIEW
published: 19 March 2021
doi: 10.3389/fonc.2021.627023

Autophagy and Cancer Dormancy

Yunus Akkoc[1†], *Nesibe Peker*[1†], *Arzu Akcay*[2] *and Devrim Gozuacik*[1,3,4*]

[1] Koç University Research Center for Translational Medicine (KUTTAM), Istanbul, Turkey, [2] Yeni Yüzyıl University, School of Medicine, Private Gaziosmanpaşa Hospital, Department of Pathology, Istanbul, Turkey, [3] Koç University School of Medicine, Istanbul, Turkey, [4] Sabancı University Nanotechnology Research and Application Center (SUNUM), Istanbul, Turkey

Metastasis and relapse account for the great majority of cancer-related deaths. Most metastatic lesions are micro metastases that have the capacity to remain in a non-dividing state called "dormancy" for months or even years. Commonly used anticancer drugs generally target actively dividing cancer cells. Therefore, cancer cells that remain in a dormant state evade conventional therapies and contribute to cancer recurrence. Cellular and molecular mechanisms of cancer dormancy are not fully understood. Recent studies indicate that a major cellular stress response mechanism, autophagy, plays an important role in the adaptation, survival and reactivation of dormant cells. In this review article, we will summarize accumulating knowledge about cellular and molecular mechanisms of cancer dormancy, and discuss the role and importance of autophagy in this context.

Keywords: autophagy, dormancy, recurrence, relapse, cancer, metastasis

OPEN ACCESS

Edited by:
Daniel Hector Grasso, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

Reviewed by:
Gabriele Multhoff, Technical University of Munich, Germany
Pablo Nicolas Fernandez Larrosa, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

***Correspondence:**
Devrim Gozuacik
dgozuacik@ku.edu.tr

[†]*These authors have contributed equally to this work and share first authorship*

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *07 November 2020*
Accepted: *22 January 2021*
Published: *19 March 2021*

Citation:
Akkoc Y, Peker N, Akcay A and Gozuacik D (2021) Autophagy and Cancer Dormancy. Front. Oncol. 11:627023. doi: 10.3389/fonc.2021.627023

INTRODUCTION

Cancer is the cause of death for millions of people every year, hence it's one of the most devastating disease. Detection and diagnosis at early stages of cancer remarkably improve the chance of cure. However, the incidence of cancer continues to rise due various factors, including tobacco use, air pollution, obesity, increased life expectancy and cancer-causing infections. First approach in the treatment of cancer is usually surgical resection of the primary tumor, often followed by chemotherapy and/or radiotherapy. Besides, recent advances in targeted therapies and immunotherapies help to reduce the tumor burden. Thanks to high resolution diagnostic tools, advances in tumor ablation techniques, drug combinations, and targeted therapeutics, 5-year survival rates are improved for some cancer types, yet overall cancer survival rates for patients suffering from advanced disease are still low. A major reason for such discrepancy is the spread of cancer cells to organs other than the primary site and formation of the metastatic lesions. In other words, metastasis is among the leading causes of cancer-related deaths.

Metastasis of cancer to distant organs requires a sequential and complex chain of events. Cancer cells need to undergo several mutations and adaptations in order to gain motility and invasiveness, intravasate (migration into vessels), survive in the blood circulation and the lymphatics, extravasate, nestle and grow at secondary sites. Metastasis and survival of cancer cells at secondary sites are also affected by "the soil" in which tumor cells are seeded, namely the tumor microenvironment or stroma (1).

Mutations promoting epithelial-to-mesenchymal transition (EMT) greatly contribute to metastasis of cancer cells. Cells of normal tissues are tightly regulated by cell-to-cell and cell-to-matrix interactions. During cancerous transformation, epithelial cells may acquire mesenchymal cell-like properties, including loss of critical epithelial markers (e.g., E-cadherin, α-catenin), and

expression of mesenchymal markers (e.g., N-cadherin and vimentin) (2). A transcriptional program orchestrates this transformation (e.g., ZEB1/2, Snail *etc.*) (3–5). Remodeling of epithelial junctions and cytoskeleton promotes motility and invasiveness of cancer cells (6). Cancer cells that are now motile and invasive, penetrate through the tissue extracellular matrix (ECM) and spread to lymph nodes and secondary sites through blood and lymph vessels. Seeding to metastatic sites and metastatic growth require reversal of this process, namely mesenchymal-to-epithelial transition (MET).

Advances in the last decade showed that in many tumor types, a small population of progenitor cancer cells, namely cancer stem cells (CSC), are responsible for the evolution and progression of the disease and metastasis (7, 8). Cancer cells and CSC might spread from primary tumors at various stages of tumor progression. These disseminated cells or clusters of cells (disseminated tumor cells, DTC) continue their evolution in their new tumor niches and they generally acquire genetic and epigenetic signatures that are different from the tumor of origin (9–13). Although aggressive proliferation of DTC might result in overt metastasis, latency periods lasting for months or even years were observed. During the latency period that spans the time between tumor formation and recurrence (also known as relapse), some cancer cells stay in a "dormant" state, a state of balanced proliferation or no proliferation at all (14, 15). At least some of these dormant cells have capacity to reactivate and form new metastatic lesions. Recurrent tumors were associated with drug resistance and aggressive behavior. So, most patients with recurrent disease show a very poor prognosis (16–18). For this reason, as an important mechanism contributing to tumor recurrence, cancer dormancy became a focus of attention in recent years.

There are two major mechanisms of cancer dormancy, namely, tumor mass dormancy and tumor cell dormancy (or cellular dormancy) (**Figure 1**). In tumor mass dormancy, proliferation of tumor cells counterbalanced by cellular demise and the tumor mass is preserved to a certain extent. A reason for limited tumor growth is hypoxia and inefficient nutrient supply due vascularization defects (angiogenic dormancy). Trimming of tumor cells by the cells of the immune system is another mechanism limiting tumor growth and expansion (immunological dormancy). On the other hand, cellular dormancy involves transition to a quiescent, cell cycle-arrest state, while cells retain the capacity to perpetuate neoplastic behavior when reactivated. In this review, we will mainly focus on the role of autophagy in cellular dormancy.

AUTOPHAGY AND CANCER DORMANCY

Mechanisms of Mammalian Autophagy

Autophagy activation was reported as a novel characteristic of dormant cells in different tumor types (19). Three major types of autophagy were described: Macroautophagy, chaperone-mediated autophagy (CMA) and microautophagy. Although a clear connection between cancer and CMA was established (20), according to our knowledge, so far no study directly connecting CMA to cancer dormancy was published. Similarly, microautophagy was not studied in this context either. On the

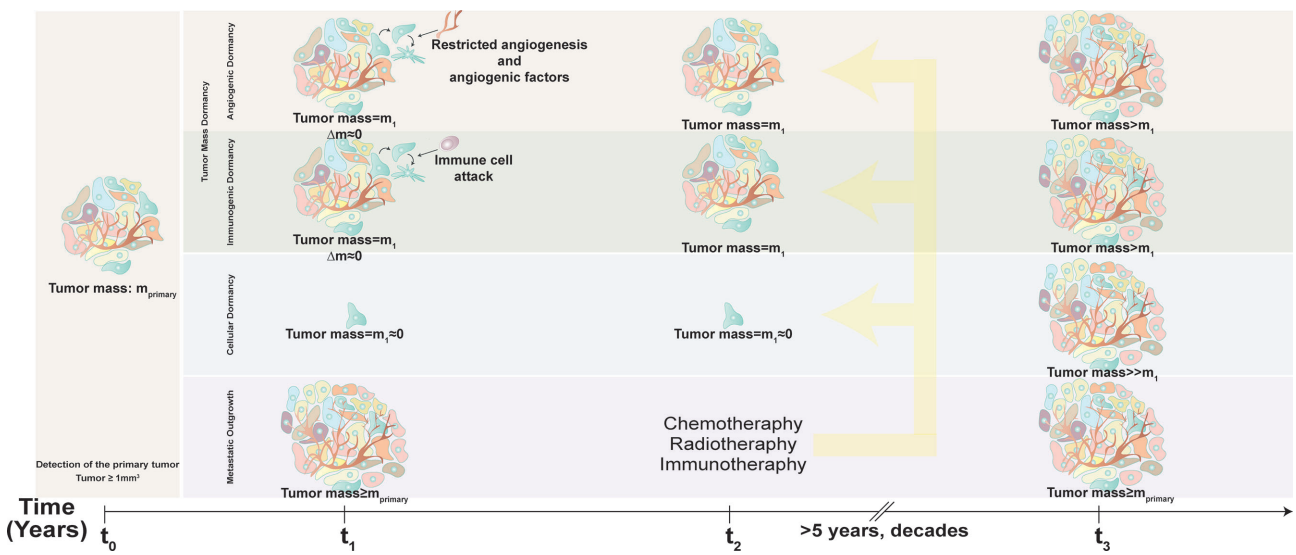


FIGURE 1 | Time-dependent progression of metastasis and dormancy. Conventional diagnostic tumor scans are able to detect tumors bigger than 1 mm^3 (tumor mass = $m_{primary}$). After diagnosis with cancer (time = t_0), patient may undergo chemotherapy, radiotherapy or adjuvant therapy, yet dormant cells escape and become resistant to these treatments (time = t_2), and awaken after years or even decades (time = t_3). In tumor dormancy, tumor mass (m_1) stagnates due to limited neovascularization and constant immune cell attack that balance tumor cell demise and proliferation. After the latency period, dormant tumor cells awaken and lead to tumor outgrowth (tumor mass>m_1). In cellular dormancy, cancer cells hibernate as single cells or small clusters (tumor mass = $m_1 \approx 0$) and lead to massive tumor growth (tumor mass≥$m_{primary}$) following exit from dormancy.

other hand, the number of studies implicating a role for macroautophagy in cancer dormancy continues to increase. Macroautophagy (autophagy herein) is an evolutionarily conserved catabolic process and an important stress response in all eukaryotic cells. Activation of autophagy leads to the clearance of various cellular components, including damaged organelles (e.g., mitochondria) as well as unfolded proteins and abnormal protein aggregates. As such, autophagy helps cells to combat stress, thereby contributes to survival. Mechanisms orchestrating autophagy activation, autophagic vesicle (autophagosome) formation and autophagic degradation were studied in detail.

Autophagic machinery primarily relies on the activity of ATG (autophagy-related) proteins (**Figure 2**). Following exposure to stress, activation of a core pathway involving ATG proteins leads to formation of double-membrane structures (phagophores) around target molecules and organelles. Phagophores eventually elongate and seal, forming closed vesicular structures called autophagosomes or autophagic vesicles. Autophagosomes fuse with late endosomes or lysosomes, to form autolysosomes. Lytic enzymes in the lumen of autolysosomes are responsible for the degradation of cargos carried by autophagosomes.

Autophagic activity is tightly controlled by protein complexes containing the mTOR kinase: mTORC1 and mTORC2 (21). These protein complexes are highly responsive to cellular cues, such as nutrient and growth factor availability, and in the active state, they work to inhibit autophagy (22). PKB/AKT pathway provides input from growth-related signals in order to regulate the mTOR complexes and autophagy. AMPK pathway, an energy sensor of the cell that monitors AMP/ATP ratios, comes into play when energy levels are low (23–25). While the mTORC1 has been documented to regulate autophagy directly, mTORC2 complex provides regulatory and feedback signals from insulin receptor phosphoinositide 3-kinase signaling (26–28). Under nutrient-rich conditions, mTORC1 keeps ULK1 and ATG13 in an inactive state. Nutrient deprivation leads to dephosphorylation of mTORC1 sites on ULK1 and ATG13 (24). ULK1 then autophosphorylates and phosphorylates its partner proteins ATG13 and FIP200 (29, 30). By this way, ULK1 activation initiates a cascade of events that promotes autophagosome formation.

Phagophore nucleation results from phosphorylation of lipids by the VPS34 lipid kinase complex (the class III PI3K, PI3KC3), BECN1 (Beclin-1), AMBRA1 and ATG14 (31, 32). Phosphorylation of inositol lipids on cellular membranes, such as ER membranes, leads to accumulation of phosphatidylinositol 3-phosphates (PI3P) (33). PI3P formation at membrane sites called omegasomes (or cradles), through recruitment of proteins with PI3P-binding domains, such as WIPI1/2 proteins and DFCP1 (34–36).

Proteins from the ubiquitin-like ATG8 family control elongation of phagophores through the activity of two key protein complexes. ATG12-ATG5–ATG16L1 complex facilitates coupling of ATG8 proteins, including MAP1LC3 (LC3) and GABARAPs, to phosphatidylethanolamine (PE) molecules on elongating membranes (37). Lipidated ATG8 proteins on autophagic membranes allow growth and closure of phagophore membranes (38, 39). Autophagy can be selective or non-selective. In the latter case, autophagy receptors, such as SQSTM1/p62, bridge between ATG8 proteins and ubiquitylated targets and direct them to autophagosomes. Hence, assessment of lipidation of ATG8s, especially LC3 lipidation, is a widely accepted as a powerful approach for monitoring autophagic activity (40, 41).

Mature autophagosomes are then transported along microtubules toward late endosomes and lysosomes. SNARE proteins facilitate fusion of autophagosomal outer membrane with endosomal or lysosomal membranes (42). Lysosomal acidic hydrolases degrade autophagic cargos into their building blocks (e.g. proteins into amino acids), which in turn are recycled to cytosol and used in the synthesis of new cellular components. As such, autophagy functions as a cellular degradation and recycling mechanism that allows cells to survive under stressful conditions.

Dysregulation of autophagy pathway is associated with various diseases, including cancer (43–45). In fact, autophagy plays an important yet context-dependent role at various stages

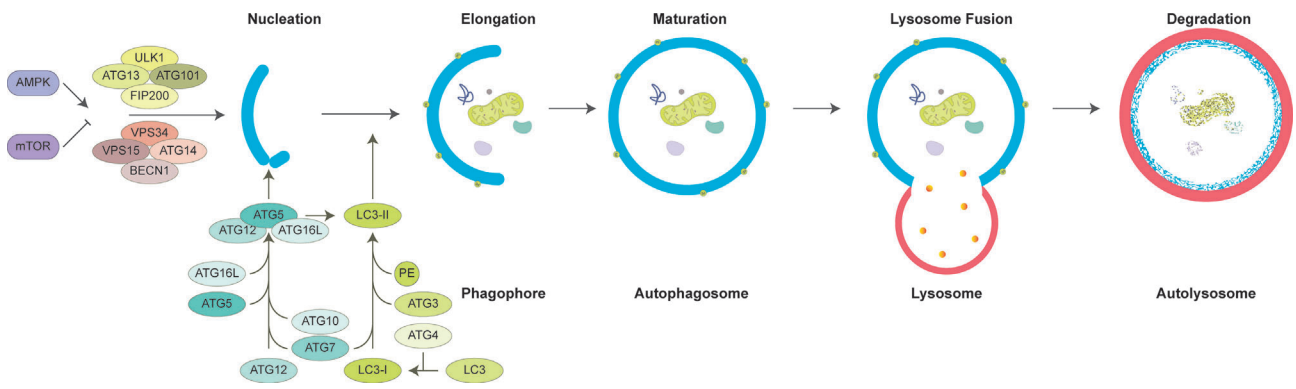


FIGURE 2 | General mechanism of mammalian autophagy. Autophagy is tightly controlled by the activity of AMPK and mTOR. Under nutrient deprivation, AMPK activates autophagy, yet mTOR inhibition is relieved. Subsequent activation of ULK1 and BECN1 complexes promotes formation of phagophore. ATG5-12-16L complex and ATG8 family protein LC3 are required for elongation and closure of phagophore. Fully mature double-layered autophagosome containing cargo molecules fuses with late endosomes and lysosomes. Autophagosomes and their cargo are degraded through lysosomal enzymes and recycled into cytosol for reuse.

of cancer progression and metastasis (45, 46). In early stages of cancer, control of ROS accumulation, prevention of DNA damage and genome instability require functional autophagic activity, eliminating damaged mitochondria and misfolded/aggregated proteins (47). Conversely, in established tumors and especially those tumors that grow relatively faster (e.g., tumors with K-RAS activation), tumor supporting role of autophagy is prominent. In this context, autophagy compensates for increased metabolic demands, originating from nutrient and energy deficiency, hypoxia and acidosis (48). Tumor stage-dependent dual role of autophagy might be explained in some models by hypoxia-triggered switches involving proteins, such as RAC3 (49). Moreover, autophagy was involved in various tumor progression- and metastasis-associated phenomena, including cell cycle regulation, stem cell behavior, extracellular matrix interactions, EMT, anoikis, tumor cell-stroma interactions, angiogenesis, immune responses and treatment resistance (50–54). In line with these observations, a number of autophagy genes and proteins show tumor suppressor or oncogenic activities (45, 55, 56).

In spite of the importance of autophagy in cancer formation and progression, contribution and molecular mechanisms of autophagy to cancer dormancy was not explored in detail in different cancer types and models. As summarized below, an increasing number of recent studies begins to provide evidence about a direct involvement of autophagy in cancer dormancy.

Mechanisms of Cellular Dormancy

Cellular dormancy is characterized by a halt in cancer cell proliferation and entrance to a quiescence-like state. This non-proliferative state of existence may last for months or years. Moreover, no matter how long the dormant state is, some cells retain the capacity to reactivate and re-enter to a proliferative state (57–59). So, cellular dormancy is defined as a reversible mechanism.

Dormant cells usually reside and survive in the G0-G1 phase of the cell cycle. Hence, they lack proliferation markers (e.g., Ki67) as well as markers of apoptosis (e.g., active-caspases) and senescence (e.g., beta-galactosidase) (60–62). Not surprisingly, several changes in cell cycle regulatory molecules were observed. For instance, cyclin-dependent kinase (CDK) inhibitors $p27^{Kip1}$ and $p21^{Cip1/WAF1}$ controlled the non-proliferative state during hematopoietic stem cell dormancy (63). In another example, adhesion of lymphoma cells to bone marrow stromal cells resulted in cell cycle arrest, involving post-transcriptional regulation of Skp2, a component of $p27^{Kip1}$ and $p21^{Cip1/WAF1}$ containing SCF complex (64).

Another regulator of dormancy-associated cell cycle arrest was identified as the DREAM complex. The complex consists of p130 or p107 (Retinoblastoma-like pocket proteins), MuvB and E2F protein. MuvB was defined as a core component in the transcriptional regulation of cell cycle genes by the DREAM complex (65). In dormant cells, elevated p130 levels were shown to facilitate DREAM complex formation and regulate its transcriptional effects (65, 66). On the other hand, high levels of p107 were detected only in proliferating cells. Regulatory kinases DYRK1A and DYRK1B phosphorylated a subunit of MuvB, namely LIN52, and activated DREAM complex assembly during entry to the non-proliferative state (67). Additionally, these kinases stabilized $p27^{Kip1}$ and induced cyclin D turnover, further contributing to the non-proliferative state (68, 69).

Mitogen-activated kinase (MAPK) pathway plays a central role in the regulation of dormancy. A number of dormancy-related factors and their cognate receptors were associated with a shift in the balance between proliferative ERK1/2 versus non-proliferative p38 MAPKs. Independent studies conducted in different cancer cell types, including breast, prostate, melanoma cells, supported the involvement of p38 pathway in cancer dormancy (70), and activation of this pathway contributed to the proliferation arrest in this context (71, 72). For instance, p38 kinases were stimulated by the activity of TGF-β2/TGFβRIII, which in turn supported dormancy of head and neck squamous cell carcinoma cells in the bone marrow (73). In addition, as a paracrine factor, secreted TGF-β2 from osteoblasts in the bone microenvironment contributed to prostate cancer dormancy through activation of p38 (74). Dormant cells secreted high levels of TGF-β2, creating an autocrine loop in the regulation of dormancy (75). In line with this, results revealed that proliferating cells have low TGF-β2 levels (75).

Urokinase plasminogen activator receptor (uPAR) pathway was described as another dormancy-associated pathway. In HNSCC, status of the uPAR was directly related with the dormancy capacity of cells *in vivo*. In this tumor type, interaction of uPAR with α5β1 integrin dictated formation of insoluble fibronectin fibrils and blocked the activation of p38 (76). Conversely, decreased uPAR levels were detected correlated with ERK1/2 pathway attenuation (76). Moreover, downregulation of uPAR inhibited focal adhesion kinase (FAK) phosphorylation and downstream Src activity, facilitating the dormant state *in vivo* (77, 78).

In addition to the HNSCC model, FAK and Src-related mechanisms were also studied in breast cancer dormancy. Activation of Src by CXCL2/CXCR4 signaling correlated with prolonged survival of DTC in the bone marrow niche *via* phosphoinositide 3-kinase (PI3K)/AKT pathway (79, 80). Interestingly, Src-assisted dormancy was secondary organ-dependent and its downregulation had no effect on lung metastasis of breast cancer cells (79, 80).

Bone morphogenetic proteins (BMP) were mainly involved in dormancy regulating tumor stroma interactions. Investigations on prostate cancer revealed that, BMP7 (bone morphogenetic protein 7) regulated dormancy of prostate cancer cells through affecting cancer stem cell population. This effect required BMPR2 (BMP receptor 2) expression and activation of dormancy-associated downstream signaling components, such as p38, p21 and the metastasis suppressor NDRG1 (81). In another study, bi-directional communication between tumor cells and stroma was revealed. Dormant prostate cancer cells, but not proliferative cells, secreted SPARC, a factor which stimulated BMP7 expression from bone marrow stromal cells, contributing to the maintenance of dormant phenotype (82). Another BMP protein, BMP4, was studied in the context of breast cancer. High levels of BMP4 expression correlated with entrance of cancer cells to a dormant state in the lung. In this organ, dormancy activation was associated with ALK2/3 and

SMAD1/5 signaling (83). In this system, extracellular BMP antagonist DAND5 counteracted BMP4-assisted dormancy and promoted the proliferative state (84).

Dormancy-related signaling pathways lead to the activation of a specific gene regulatory program. For instance, BHLHE41 transcription factor was documented among the downstream effectors of p38-regulated dormancy in HNSCC (72). In another study, Kim et al. identified BHLHE41 and NR2F1 as key factors promoting ER positive breast cancer dormancy in an *in vivo* xenograft mice model (85). Importance of BHLHE41 to breast cancer dormancy was further confirmed in a 3D endosteal bone niche model containing bone marrow-derived cells and endothelial cells (86).

NR2F1 belongs to the NR2F family of cancer-related transcription factors (77). Dormant cancer cells were found to express high levels of NR2F1 in comparison to their proliferative counterparts (79, 87). Moreover, high NR2F1 and TGF-β2 expression were characterized as a dormancy signature in prostate cancer DTC (87). Moreover, transcription of another p38-regulated gene, SOX9, was directly controlled by NR2F1 binding elements in its promoter (87). NR2F1-SOX9 axis was also regulated by microenvironment-derived retinoic acid (RA) signaling and RARβ (87). In addition to other targets, NR2F1 promoted expression of the CXCL12 and its receptor CXCR4 and induced-cell cycle arrest in salivary adenoid cystic carcinoma cells (79).

Receptor tyrosine kinases, including TYRO3, AXL and MER, were critically involved in the dormancy phenotype of certain cancer types. For example, activation of AXL or TYRO3 receptor kinases by GAS6 secreted from osteoblast cells, contributed to the establishment of metastatic dormancy of prostate cancer cells in the bone marrow (75, 88, 89). In another example, dormant state was triggered in lymphoblastic leukemia cells by GAS6 to MER binding (90). On the other hand, AXL was found to be an important regulator of myeloid lineage-related gene expression and dormancy in myeloma cells (91).

Although generally considered as a pathway involved in cancer dissemination and metastasis (92), Wnt pathway was implicated in dormancy control in a context- and stimulus-dependent manner (93, 94). For instance, DKK1-dependent inhibition of Wnt3a signaling induced growth arrest and entry to dormancy (95). On the other hand, activation of Wnt5a pathway was responsible for the entrance of prostate cancer cells to a non-proliferative dormant state (94).

Overall, several cytokines, growth factors and signaling pathways involving kinases as well as transcription factors were identified as regulators of dormancy. Although we are far from having a complete picture, pathways regulating dormancy are being better defined. A summary of known proteins and pathways studied *in vitro* and *in vivo* were shown in **Tables 1** and **2** respectively, and the reader is referred to recent review articles about dormancy for further details (58, 191, 192).

Role of Autophagy in the Context of Cancer Dormancy

In addition to recycling long-lived proteins, autophagy plays a key role in the management of energy crisis, control of reactive oxygen accumulation through destruction of damaged mitochondria, and in the elimination of unfolded and misfolded proteins. Studies in the last decade indicated that autophagy is involved in various stages of cancer formation and progression (193–197). As mentioned previously, autophagy plays a role in various events leading to tumor cell survival, resistance to treatment and metastasis. Hence, autophagy emerges as one of the critical determinants of the dormant state. In fact, several independent studies using cancer cells-derived from a wide variety cancer types, including breast, ovary, gastrointestinal tract, pancreas and bone cancers and their respective mice tumor or xenograft models showed that, autophagy is highly active in dormant cancer cells (125, 148, 180, 198–200). Some of these observations were even supported by the analysis of patient-derived tissue samples (201), yet molecular details of how and why autophagy contributes to the dormant phenotype are not well known. In this section, we will overview the current literature on autophagy-dormancy connection.

Autophagy-Dormancy Connection: Experimental Evidence

Studies using different experimental set-ups, different cancer cell types and models revealed that, malignant cells entering a non-proliferative, dormancy-like but reversible cycle arrest state showed increased autophagic activity (**Table 3**) (148, 180, 199). In this context, dormant cancer cells were more sensitive to autophagy inhibition compared to their proliferating counterparts and inhibition of autophagy was lethal in most cases. Moreover, inhibition of autophagy in dormant cancer cells changed their metastatic behavior *in vivo* in mice (148, 199).

For instance, in gastrointestinal stromal tumor (GIST) cells, treatment with a KIT/PDGFRA inhibitor, imatinib, induced a dormancy-like quiescent state during which cells entered cell cycle arrest through accumulation of the cell cycle inhibitor p27 (198). Autophagy activation was observed under these conditions, and inhibition of autophagy using a genetic or chemical (chloroquine or quinacrine treatment) approach resulted in the loss of cell viability, and increased the anti-tumor efficacy of imatinib in *in vitro* and *in vivo* tests.

Contribution of autophagy to ovarian cancer dormancy was studied in detail. DIRAS3 (or ARHI) is a maternally imprinted tumor suppressor that is frequently downregulated in breast and ovarian cancers (209, 210). Re-expression of DIRAS3 in cancer cells robustly induced autophagy (180, 202, 211). Interestingly, although DIRAS3 expression resulted in the apoptotic death of cancer cells in culture (203), it promoted a dormancy-like state *in vivo* (180, 202). Re-expression of DIRAS3 in a Tet-inducible manner, stimulated autophagy in ovarian cancer xenografts, and led to a reversible inhibition of tumor growth and entry to a dormant state. Downregulation of the tumor suppressor was sufficient for the establishment of overt metastatic tumors. In this model, inhibition of DIRAS3-induced autophagy by chloroquine (a lysosomal autophagy inhibitor) reduced tumor growth, further underscoring the importance of autophagic activity to DIRAS3-related dormancy (202).

TABLE 1 | Summary of *in vitro* dormancy models and mechanisms.

	Proteins and Factors	Cell line	Tissue of Origin	Metastatic Target	Dormancy Tests	Effect on Dormancy	Dormancy Mechanism	Reference
1	Osteopontin	Nalm-6	ALL	Bone	Ki67 positivity, Fluorescent dye retaining (DiR), Drug resistance (Ara-C)	Induction	Tumor niche	(96)
2	Bcl-2	CD34-enriched primary AML	AML	N.D.	Drug resistance (Ara-C), Fluorescent dye retain (PKH26),	Induction	Tumor niche, apoptosis	(97)
3	FGF-2	T47D, MCF7	Breast	N.D.	Drug resistance (Taxotere)	Induction	Integrin and PI3K/Akt signaling	(98)
4	CK19	BT549, MDA-MB-231	Breast	N.D.	G0/G1 cell cycle arrest, Drug resistance (Cisplatin)	Induction	p38 signaling, ER stress	(99)
5	ΔNp63a	MCF7	Breast	N.D.	3D spheroid formation, G0/G1 cell cycle arrest, Drug resistance (Paclitaxel, Doxorubicin), Ki67and BrdU positivity	Induction	Wnt signaling	(100)
6	NR2F1-AS1	BT474	Breast	Lung	Ki67 positivity, Colony formation	Induction	Her2/neu and ER/PR hormone receptor signaling	(101)
7	p21	MCF10A	Breast	N.D.	BrdU positivity, G0/G1 cell cycle arrest	Induction	CDK2 signaling	(102)
8	miR-222/223	MDA-MB-231, T47D	Breast	Bone	G0/G1 cell cycle arrest, Drug resistance (Carboplatin), Stem-like phenotype (Oct4+)	Induction	CDKIs	(103)
9	IKKβ	MCF7	Breast	Bone, pelvic organs, lung	Colony formation, G0/G1 cell cycle arrest, Ki67 positivity	Induction	CDKIs, Stemness	(104)
10	BHLHE41, Wnt3, HBP1	MDA-MB-231	Breast	N.D.	3D spheroid formation	Induction	Tumor niche, p38/ERK signaling	(77)
11	IL1β	T47D, MCF7	Breast	N.D.	Drug resistance (Fulvestrant), colony formation, G0/G1 cell cycle arrest, p-p38/p-ERK1/2 ratio	Induction	Tumor niche, p38/ERK signaling, CDKIs	(105)
12	Fra-1	4TO7	Breast	N.D.	Drug resistance (Doxorubicin, Cyclophosphamide), G0/G1 cell cycle arrest, Stem-like phenotype (Sca-1+), Ki67 positivity	Induction	N.D.	(106)
13	LOXL2	MCF7	Breast	N.D.	Stemness (CD44 high/CD24low), 3D matrigel spheroid formation	Inhibition	EMT, Stemness	(107)
14	MLCK	D2.A1, D2OR, MCF7, MDA-MB-231	Breast	N.D.	3D spheroid formation	Inhibition	Integrin signaling, FAK, CDKIs	(108)
15	Src	D2.0R	Breast	Lung	Ki67 positivity, G0/G1 cell cycle arrest, 3D spheroid formation	Inhibition	ERK1/2 signaling, CDKIs	(109)
16	Profilin-1	MDA-MB-231	Breast	N.D.	3D matrigel spheroid formation	Inhibition	SMAD, FAK and ERK signaling	(110)
17	Parathyroid hormone-related protein (PTHrP)	MCF7	Breast	N.D.	RNA-seq dormancy associated gene downregulation (e.g. SOCS3, AMOT)	Inhibition	Ca+2 signaling	(111)
18	CXCL5	PyMT	Breast	Bone	Ki67 positivity	Inhibition	CXCL5/CXCR2 signaling	(112)
19	RhoA/RhoC	MCF-7, MDA-MB-231	Breast	N.D.	Ki67 positivity, Colony formation	Inhibition	ECM, JNK/SAPK signaling	(113)
20	Zeb1	D2A1, 67NR, 168 FARN	Breast	Lung, Bone, Adrenal gland	Fluorescent dye retaining (CFSE)	Inhibition	Immunogenic response	(114)
21	ZEB2	SW480	Colorectal	N.D.	Fluorescent dye retaining (PKH26)	Induction	EMT, CDKIs, Stemness	(115)
22	SDF-1α	HT-29, SW480	Colorectal	N.D.	Drug resistance (5-fluorouracil, irinotecan, oxaliplatin)	Induction	Tumor niche	(116)
23	IL-23/IL-23R	TE-1, ECA 109	Esophagus	N.D.	G0/G1 cell cycle arrest, Stem-like phenotype (CD133+), p21 and p16 expression, Radioresistance	Induction	Stat3/Wnt/Notch signaling	(117)
24	CXCL12, CXCL16 and CX3CL1	LN229, T98G	Glioblastoma	N.D.	Drug resistance (Temozolomide), Ki67 positivity, Fluorescent dye retaining (DiO), p-p38/p-ERK1/2 ratio	Induction	N.D.	(118)
25	PP2A	Primary Tumor stem-like cells (TSCs)	Glioblastoma	N.D.	G0/G1 cell cycle arrest, BrdU positivity	Inhibition	AKT and p53 signaling	(119)
26	Aurora kinase A (AURKA)	Hep2	Larynx	N.D.	Drug resistance (5-FU), G0/G1 cell cycle arrest,	Induction	CDKIs	(120)

(Continued)

TABLE 1 | Continued

	Proteins and Factors	Cell line	Tissue of Origin	Metastatic Target	Dormancy Tests	Effect on Dormancy	Dormancy Mechanism	Reference
27	miR-122	HCC-BCLC9 stem-like cell	Liver	N.D.	G0/G1 cell cycle arrest, p-p38/p-pERK1/2 ratio	Induction	Smad-independent TGF-β signaling, FOXO1, FOXO3A, MYC and AKT signaling	(121)
28	p53	A549, H460	Lung	N.D.	3D matrigel spheroid formation, Drug resistance (5-FU), G0/G1 cell cycle arrest, EdU positivity	Induction	TGF-β/smad-slug signaling, CDKIs, stemness	(122)
29	3D fibrin matrix stiffness	B16F10	Melanoma	N.D.	Ki67 positivity, PCNA positivity, G0/G1 cell cycle arrest	Induction	Integrin signaling, FAK, CDKIs	(123)
30	(TRP)-2	Prominin-1 (CD133)+ RET transgenic primary mouse melanoma cells	Melanoma	Bone	Ki67 and PCNA positivity	Induction	Immunogenic response	(124)
31	IGF2	AXT	Osteosarcoma	N.D.	G0/G1 cell cycle arrest, Ki67 positivity, Drug resistance (Adriamycin)	Induction	PI3K/Akt pathway, Autophagy	(125)
32	Dyrk1A	iOvCa147E2 and HEY	Ovary	N.D.	Cell cycle analysis by FACS, Drug resistance (Carboplatin)	Induction	CDKIs	(126)
33	AKT	OVCAR3, primary Epithelial ovarian cancer (EOC)	Ovary	N.D.	Ki67 and BrdU positivity, G0/G1 cell cycle arrest, 3D sphere formation	Inhibition	AKT signaling, CDKIs	(127)
34	NUP62	TOV112D	Ovary	N.D.	G0/G1 cell cycle arrest, Drug resistance (Cisplatin)	Inhibition	Nuclear pore architecture	(128)
35	TBK1	FGβ3	Pancreas	N.D.	Drug resistance (Erlotinib), 3D spheroid formation	Induction	Integrin signaling, αvβ3-KRAS-NF-κB axis	(129)
36	AKT	AsPC-1	Pancreas	N.D.	BrdU positivity, Drug resistance (5FU, SN38), G0/G1 cell cycle arrest, 3D matrigel spheroid formation	Inhibition	Tumor metabolism	(130)
37	Axl	PC3, DU145	Prostate	Bone	BrdU and Ki67 positivity, Fluorescent dye retaining (DiD)	Induction	TGFβ signaling	(75)
38	IRF7	RM1	Prostate	Bone, Lung	Fluorescent dye retaining (PKH26)	Induction	Immunogenic response, Type I IFN pathway (IFNAR)	(131)
39	MLCK	LuCaP 86.2, 92, and 93	Prostate	N.D.	Ki67 positivity	Inhibition	ECM, TGFβ signaling	(132)

TABLE 2 | Summary of *in vivo* dormancy models and mechanisms.

	Proteins and Factors	Cell line	Tissue of origin	Metastatic target	Dormancy tests	Effect on dormancy	Dormancy mechanism	Reference
1	Notch3 ICD	MOLT-3, MICOL-14	ALL, Colorectal	N.D.	Xenograft tumor formation	Inhibition	Tumor niche	(133)
2	CXCL10	DA1-3b cell line	AML	Bone, spleen and liver	Allograft tumor formation ratio	Induction	Immunogenic response	(134)
3	ILK	J82, JB-V	Blader	N.D.	Xenograft tumor formation, Ki67 positivity	Inhibition	ECM	(135)
4	MMP2	Dunn, LM8	Bone	Liver, kidney, lung	Allograft tumor formation, 3D matrigel spheroid formation	Inhibition	Integrin signaling	(136)
5	miR-200b/ 200a/429 cluster	RJ345	Breast	Lung	3D matrigel spheroid formation, Xenograft tumor formation	Induction	EMT	(137)
6	POSTN	MDA-MB-231, T4-2	Breast	Bone, Brain, Lung	Kİ67 positivity, Allograft tumor formation	Induction	Angiogenesis, Tumor niche	(138)
7	MSK1	T47D	Breast	Bone	Ki67 and BrdU positivity, Xenograft tumor formation	Induction	p38 signaling, GATA3/ FOXA1 axis	(139)
8	LPA1	4T1, MDA-MB-231T	Breast	Liver, Lung	Xenografted tumor formation, Ki67 positivity	Induction	p38, FAK, PLCβ signaling	(140)
9	Int2/Fgf3	MMTV(LA)-Induced Mammary Tumor	Breast	N.D.	Xenograft tumor fomation, BrdU positivity	Induction	Wnt signaling	(141)
10	Notch-2	MDA-MB-231	Breast	Liver	Ki67 and phospho-Histone H3b positivity, Drug resistance (Doxorubicin), Xenograft tumor formation, Stem-like phenotype (Sca1, CD34+)	Induction	Tumor niche, Notch signaling	(142)
11	TIE2	MCF7, 4T1	Breast	Bone	G0/G1 cell cycle arrest, Xenograft tumor formation, Drug resistance (5-FU)	Induction	Tumor niche, CDKIs	(143)
12	CXCR4	MDA-MB-231	Breast	Lung	G0/G1 cell cycle arrest, Ki67 positivity, Xenograft tumor formation	Induction	Tumor niche	(144)
13	Ron	PyMT-MSP	Breast	Lung	Allograft tumor formation	Induction	Immunogenic response	(145)
14	Fbxw7	E0771 and MDA-MB-231	Breast	Bone	Xenograft tumor formation, Allograft tumor formation, Drug resistance (Paclitaxel), Fluorescent dye retaining (PKH26), G0/G1 cell cycle arrest, 3D spheroid formation, Ki67 positivity	Induction	N.D.	(146)
15	BHLHE41, NR2F1	MCF7	Breast	N.D.	Xenograft tumor formation	Induction	N.D.	(85)
16	HSP27	MDA-MB-436	Breast	N.D.	Xenograft tumor formation, Ki67 positivity	Inhibition	Angiogenesis	(147)
17	Pfkfb3	D2.OR, D2.A1 cells	Breast	Lung	Xenograft tumor formation, 3D matrigel spheroid formation	Inhibition	Autophagy	(148)
18	FGFR1	Wnt1/ iFGFR1-driven breast cancer cell	Breast	N.D.	Xenograft tumor formation, Ki67 positivity	Inhibition	EGFR signaling	(149)
19	HER2/neu	MMTV-rtTA;TetO-NICD1 cells	Breast	N.D.	Colony formation, Tumor formation	Inhibition	Notch signaling	(150)
20	miR-205, miR-31	MDA-MB-231	Breast	Bone, brain and lung	Xenograft tumor formation, 3D sphere formation	Inhibition	Tumor niche, UBE2N/ Ubc13 signaling	(151)
21	ROCK1	MDA-MB-231	Breast	N.D.	Xenograft tumor formation	Inhibition	Tumor niche	(152)
22	VCAM1	SCP6, TM40D, MCF7, CN34, MDA-MB-435	Breast	Bone	Xenograft tumor formation	Inhibition	Tumor niche, Integrin and NFκB signaling	(153)
23	Coco	4TO7, 4T1	Breast	Bone, Brain, Lung	Ki67 and EdU positivity, Allograft tumor formation, Fluorescent dye retaining (PKH26), 3D spheroid formation	Inhibition	Tumor niche, SMAD pathway, Stemness	(84)
24	Angiopoietin-2	MCF7	Breast	N.D.	3D matrigel spheroid formation, Xenograft tumor formation, p-p38/p-ERK 1/2 ratio,	Inhibition	Tumor niche	(154)
25	IRF7	4T1	Breast	Lung	Xenograft tumor fromation, Drug resistance (Methotrexate, Doxorubicin),	Induction	Immunogenic response, Type I IFN pathway (IFNAR)	(155)
26	ERK/p38	MDA-MB-231, MCF7, Hep3, M24met	Breast, Head and neck, Melanoma	N.D.	Xenograft tumor formation on CAM (Chorioallantoic membrane)	Induction	p38/ERK and Integrin signaling	(70)

(Continued)

TABLE 2 | Continued

	Proteins and Factors	Cell line	Tissue of origin	Metastatic target	Dormancy tests	Effect on dormancy	Dormancy mechanism	Reference
27	H2BK, Eph receptor A5 (EphA5), Angiomotin	MDA-MB-436, KHOS-24OS, T98G, SW872	Breast, osteosarcoma, glioblastoma, liposarcoma	N.D.	Xenograft tumor formation	Induction	Angiogenesis	(156)
28	CUL4B	Patient-derived tumor organoid (PDOs) cell, HT29 and HCT116	Colorectal	Liver, Lung	3D matrigel spheroid formation, Xenograft tumor formation	Induction	Epigenetic alteration, Stemness	(157)
29	TSP-1, EGFR	U87-MG, T98G	Glioblastoma	N.D.	Xenograft tumor formation, Drug resistance (Erlotinib, Cetuximab), 3D matrigel spheroid formation	Induction	Angiogenesis, EGFR signaling	(158)
30	Tissue factor (TF)	U373	Glioblastoma	N.D.	Xenograft tumor formation, Ki67 positivity	Inhibition	Immunogenic response	(159)
31	miR-190	T98G, KHOS-24OS	Glioblastoma, Osteosarcoma	N.D.	Ki67 positivity, Xenograft tumor formation	Induction	Immunogenic response, antigen presenting	(160)
32	PRRX1	Cal-27, SCC-9	Head and neck	N.D.	Xenograft tumor formation	Induction	EMT, TGF-β and p38 signaling	(161)
33	α5β1 Integrin	HEp3	Head and neck	N.D.	Xenograft tumor formation on CAM (Chorioallantoic membrane), G0/G1 cell cycle arrest	Induction	p38 and Integrin signaling	(162)
34	ATF6a	D- and T-variant of HEp3	Head and neck	N.D.	Xenograft tumor formation on CAM (Chorioallantoic membrane)	Induction	UPR, Rheb-mTOR and MKK6/p-38 axis	(163)
35	BHLHE41	Hep3	Head and neck	N.D.	Xenograft tumor formation	Induction	p53, c-Jun signaling	(72)
36	Aurora kinase A (AURKA)	Hep2	Head and neck	Lung	Xenograft tumor formation, G0/G1 cell cycle arrest, Ki67 positivity	Inhibition	FAK/PI3K/Akt signaling	(164)
37	TGFβ2	Hep3	Head and neck	Lung, Bone	p-p38/p-ERK ratio, Xenograft tumor formation	Induction	SMAD pathway, CDKIs	(73)
38	NR2F1	Hep3	Head and neck	Spleen, Lung	Ki67 positivity, Xenograft tumor formation	Induction	Epigenetic alteration, Retinoic acid pathway, Stemness	(87)
39	Fibrinogen fibrils	HEp3	Head and neck	N.D.	Xenograft tumor formation on CAM (Chorioallantoic membrane)	Induction	p38/ERK and Integrin signaling	(76)
40	MYC	LAP-tTA Tet-o-MYC cells	Liver	N.D.	Ki67 positivity, Xenograft tumor formation	Inhibition	N.D.	(165)
41	YAP/TEAD	PC-9	Lung	N.D.	Xenograft tumor formation, Drug resistance (Osimertinib+ Trametinib)	Induction	EMT, Evasion of apoptosis	(166)
42	PAX5	Raji	Lymphoblastoid	N.D.	EdU, Fluorescent dye retaining (CFSE), G0/G1 cell cycle arrest, Xenograft tumor formation, Drug resistance (Etoposide, Daunorubicin)	Inhibition	N.D.	(167)
43	KISS1	C8161.9	Melanoma	Lung, Bone, Kidney, Eye	Xenograft tumor formation	Induction	Ca^{+2} and AKT signaling	(168)
44	Sox2	B16F1, A375	Melanoma	N.D.	3D fibrin spheroid formation, Ki67, COUP-TF1 and BrdU positivity, G0/G1 cell cycle arrest, Drug resistance (Tazarotene, ATRA, Temozolomide, Cisplatin), Stemness (CD133+) Xenograft tumor formation	Induction	Retinoic acid pathway, STAT3 and p53 signaling, CDKIs	(169)
45	IFN-γ	B16, A375	Melanoma	N.D.	3D spheroid formation, Xenograft tumor formation, G0/G1 cell cycle arrest, PCNA positivity, Drug resistance (Methotrexate, paclitaxol)	Induction	ECM, IDO1/AhR-dependent p27 pathway STAT1 pathway	(170)
46	Angiostatin	B16F10	Melanoma	Lung	Xenograft tumor formation	Induction	N.D.	(171)
47	VEGF	B16F10	Melanoma	N.D.	Xenograft tumor formation	Inhibition	Angiogenesis	(172)
48	GILZ	B16F1, B16F1-GM-CSF	Melanoma	Brain	Stemness (CD133, CD24 positivity), Xenograft tumor formation, G0/G1 cell cycle arrest	Inhibition	FOXO3A signaling, CDKIs	(173)

(Continued)

TABLE 2 | Continued

	Proteins and Factors	Cell line	Tissue of origin	Metastatic target	Dormancy tests	Effect on dormancy	Dormancy mechanism	Reference
49	EET	LLC, B16F10,T241	Melanoma, Sarcoma	Lung, axillary lymph nodes, liver and kidney	Allograft tumor formation	Inhibition	Tumor niche, angiogenesis	(174)
50	TIMP-1 and TIMP-2	MLS 402-91 and primary human myxoid liposarcoma	Myxoid liposarcoma	N.D.	Xenograft tumor formation	Induction	Angiogenesis, lipogenesis	(175)
51	LTBP2	HONE1-2, NP460	Nasopharynx	N.D.	Colony formation, 3D matrigel spheroid formation, Xenograft tumor formation	Induction	N.D.	(176)
52	miR-34a, miR-93, and miR-200c	Saos-2, MG-63	Osteosarcoma	Lung	Ki67 positivity, Xenograft tumor formation	Induction	Angiogenesis, EMT	(177)
53	ARHI (DRAS3)	SKOv3, Hey	Ovary	N.D.	Xenograft tumor formation, Colony formation, PCNA positivity	Induction	Angiogenesis, Epigenetic alterations	(178)
54	VEGF, IL8 and IGF-1	SKOv3, OVCAR8	Ovary	N.D.	Xenograft tumor formation	Induction	Autophagy, ERK and AKT signaling	(179)
55	ARHI (DIRAS3)	SKOv3	Ovary	N.D.	Colony formation, Xenograft tumor formation	Induction	PI3K and TSC1/2 signaling, autophagy	(180)
56	MED12	HO8910 and SKOV3	Ovary	N.D.	Xenograft tumor formation, Colony formation, G0/G1 cell cycle arrest, Drug resistance (paclitaxel, gemcitabine, topotecan, and 5-FU)	Inhibition	EGFR signaling	(181)
57	CXCR4	A2780, SKOv-3	Ovary	N.D.	Drug resistance (cisplatin, doxorubicin, paclitaxel), Xenograft tumor formation, Colony formation	Inhibition	Tumor niche, EMT	(182)
58	IGF1	AsPC-1, MIA PaCa-2	Pancreas	N.D.	Allograft tumor formation, Xenograft tumor formation, G0/G1 cell cycle arrest, Ki67 positivity	Induction	IGF1/IGF-1R/AKT/XIAP signaling axis	(183)
59	IL8	R254, H6c7-kras, Panc1	Pancreas	N.D.	Ki67 positivity, p-P38/p-PERK1/2 ratio, Xenograft tumor formation	Induction	Tumor niche, Cytokine signaling	(184)
60	15-LOX-2	DU145, PC-3	Prostate	N.D.	Xenograft tumor formation, G0/G1 cell cycle arrest	Induction	Angiogenesis	(185)
61	MERTK	PC3, C4-2B	Prostate	Bone	Xenograft tumor formation, G0/G1 cell cycle arrest, Ki67 positivity	Induction	p38/ERK signaling, Stemness	(186)
62	BMP-7	PC3mm	Prostate	Bone	Xenograft tumor formation, Stemness (CD24low/CD44high/CD133high), Fluorescent dye retaining (DiD)	Induction	p38 signaling, SPARC/ BMP7/BMPR2 axis, CDKIs	(82)
63	BMP7	PC3 mm, C4-2B	Prostate	Bone	3D sphere formation, Xenograft tumor formation, Stemness (CD24-/CD44 +/CD133+)	Induction	BMPR2/NDRG1/P38 axis	(187)
64	GDF10, TGFβ2	C4-2B4	Prostate	Bone	Ki67 positivity, Xenograft tumor formation, p-p38/p-ERK1/2 ratio	Induction	Tumor niche, TGFβRIII-p38-pS249/T252 RB signaling axis	(74)
65	Axl	PC3, C42B	Prostate	Bone	Xenograft tumor formation	Induction	Tumor niche, platelet aggregation and activation	(188)
66	Axl, Tyro3	PC3, Du145	Prostate	Bone	Xenograft tumor formation	Induction	Tumor niche	(89)
67	Anxa2	PC3	Prostate	N.D.	Xenograft tumor formation, G0/G1 cell cycle arrest	Induction	Tumor niche	(90)
68	TBK1	PC3, C4-2B	Prostate	Bone	Xenograft tumor formation, Drug resistance (Taxotere)	Induction	Tumor niche, mTOR signaling	(189)
69	Wnt5a	PC3, C4-2B	Prostate	Bone	Fluorescent dye retaining (DiD), Xenograft tumor formation, Drug resistance (Docetaxel), G0/G1 cell cycle arrest, Ki67 positivity	Inhibition	Tumor niche, Wnt5a/ ROR2/SIAH2 signaling axis, CDKIs	(94)

(Continued)

TABLE 2 | Continued

	Proteins and Factors	Cell line	Tissue of origin	Metastatic target	Dormancy tests	Effect on dormancy	Dormancy mechanism	Reference
70	Angiostatin	PC-3, Colon A, MDA-MB-231; Lewis Lung, T241, M5076	Prostate, Colon, Breast, Lung, Fibrosarcoma, Sarcoma	N.D.	Xenograft tumor formation, Ki67 staining	Induction	Angiogenesis	(190)
71	NR2F1	SACC-83, SACC-LM	Salivary gland	Lung	G0/G1 cell cycle arrest, Xenograft tumor formation	Induction	CXCL12/CXCR4 signaling	(79)

In other ovarian cancer studies, ascites-derived primary cancer cells from patients with high-grade serous ovarian cancer and ovarian cancer cell lines were used. Treatment of these cells with an allosteric AKT inhibitor Akti-1/2 induced a dormancy-like cytostatic response, and under these conditions, autophagic activity was significantly increased (204).

The role of autophagy in dormancy was studied in detail in breast cancer cell culture and animal models. Autophagy activation was observed during a dormancy-like arrest state of MCF7 breast cancer cells that were cultured with farnesyl transferase inhibitors (FTIs) (212). In MDA-MB-231 breast cancer cells, repetitive long-term hypoxia/reoxygenation cycles resulted in a low proliferation state and dormancy-like reversible cell cycle arrest (206). In another study, a dormancy-like state was induced by an adriamycin- (ADR-) treatment *in vitro* regimen using breast cancer cells from a Neu-driven cancer mice model (FVBN202 mice). Autophagy activation was also observed in this model of dormancy (207).

Other groups used two breast cancer cell lines derived from murine mammary hyperplastic alveolar nodules for modeling dormant versus proliferation states of this cancer type. D2.A1 cells were metastatic and D2.0R cells were dormancy-prone under certain growth conditions. In this system, autophagic activity of dormant D2.0R cells was found to be significantly higher than that of D2.A1 metastatic cells (148, 199). Both autophagosome and autolysosome numbers were increased, autophagy receptor (e.g. SQSTM1/p62) degradation was observed, indicating that autophagy in dormant cells was fully functional (148). In line with studies in other cancer types, dormant breast cancer cells were sensitive to autophagy inhibition whereas proliferative cells were resistant (199). Following tail vein injection to mice, most D2.0R cells stayed dormant in the lungs. Autophagy-related gene expression and autophagic activity in micrometastatic dormant lesions of D2.OR cells were observed higher as compared to the metastatic lesions of D2.A1 cells (148, 199).

Dormancy was also investigated in pancreatic duct adenocarcinoma (PDAC). In this cancer type, elevated levels of copper were associated with the degree of cancer progression. Interestingly, blockage of copper absorption by targeting the copper transporter 1 (SLC31A1) or usage of copper chelator tetrathiomolybdate (TM) inhibited proliferation of cancer cells and induced a dormancy-like arrest state (200). Under these conditions, autophagy was activated, and it was responsible for PDAC cell survival both *in vitro* and *in vivo* tests. Indeed, inhibition of autophagy caused an increase in *in vitro* cell death and decreased *in vivo* tumor burden. These results further provided evidence about the role of autophagy in the survival of dormant cells.

Dormancy in osteosarcoma following chemotherapy, has been associated with increased levels of IGF2 (125). Chronic exposure of osteosarcoma cells to IGF2 or insulin in combination with serum deprivation, successfully established an *in vitro* dormancy and drug-resistance model in osteosarcoma (125). Under these conditions, autophagy was activated.

Analysis of patient-derived samples provided further evidence about the importance of autophagy in cancer dormancy (201). In primary ovarian tumor tissue sections,

TABLE 3 | Dormancy models with documented autophagic activity.

	Dormancy activating conditions	Cell line	Cancer type	Status of autophagy	References
1	Re-expression of DIRAS3/ ARHI	SKOv3	Ovarian cancer	Activated	(180, 202, 203)
2	Akt1/2 inhibition	Ascites-derived primary human cancer cells	Ovarian cancer	Activated	(127, 204)
3	LKB1	Ascites-derived primary human cancer cells	Ovarian cancer	Activated	(205)
4	Farnesyltransferase inhibitors (FTIs)	MCF7	Breast cancer	Activated	(113)
5	Hypoxia/Re-oxygenation	MDA-MB-231	Breast cancer	Activated	(206)
6	Adriamycin-(ADR-) treatment	Neu-derived mammary cancer cells/mice model (FVBN202 mice)	Breast cancer	Activated	(207)
7	ECM	D2.A1, D2.0R	Breast cancer	Activated	(199)
8	Pfkfb3	D2.A1, D2.0R	Breast cancer	Activated	(148)
9	SYK inhibitor, R406	4T1	Breast cancer	Activated	(208)
10	SLC31A1 orT etrathiomolybdate (TM)	Panc-1, MiaPaCa-2	Pancreas cancer	Activated	(200)
11	IGF2 or Insulin	c-MYC in bone marrow stromal cells derived from Ink4a/Arf knockout mice cells (AXT)	Osteosarcoma	Activated	(125)
12	KIT/PDGFRA inhibitor, imatinib	GIST-T1	Gastrointestinal stromal tumor (GIST)	Activated	(198)

LC3 localization in punctate structures was observed in only 21–23% of cases. In contrast, LC3 puncta, hence an upregulation of the autophagic activity was observed in more than 80% of tumor nodules found on the peritoneal surface of patients at second-look operations following primary chemotherapy. These results point out to a significant increase in autophagy in dormant ovarian cancer cells seeded in the peritoneum compared to primary tumor samples. These results underline the relevance and importance of experimental observations about dormancy-autophagy connection.

Role of Autophagy in Dormancy Establishment, Dormant Cell Survival, and Reactivation

Autophagy controls dormant cell survival and behavior in many ways. In DIRAS3-induced ovarian cancer dormancy model, ARHI re-expression enabled SKOv3 ovarian cancer cells to remain dormant when they were grown in mice as xenografts (180). Reduction of ARHI levels in dormant cells caused xenografts to grow faster, and inhibition of ARHI-induced autophagy with chloroquine dramatically blocked regrowth of tumors.

In the D2.0R dormant and D2.A1 metastatic breast cancer cell models, autophagy was critical for the maintenance of the dormant phenotype in cancer cells and their survival. In 3D cultures, dormant D2.0R cells lost viability following treatment with autophagy inhibitors hydroxychloroquine, bafilomycin or 3-methyladenine, while non-dormant counterparts were not affected (199). Knockdown of autophagy genes Atg3, Atg7, p62 or FIP200, resulted in the outgrowth of dormant cells in 3D cell cultures. Moreover, Atg3-deficient D2.0R cells showed an increased capacity to create pulmonary tumors in mice (148). Similarly, in the ADR-induced dormancy model of Neu-driven breast cancer, mice that were *i.v.* injected with ADR-treated Atg5 knockdown cancer cells developed lung metastasis significantly sooner than those that were injected with wild-type dormant cells. As expected, a higher frequency of Ki67 positive, polyploid-like cells was observed in ADR-treated Atg5 knockdown mammary tumors (207). In line with these results, autophagy was downregulated in proliferating metastatic cells, but it was found to be necessary for a dormant-to-proliferative switch before the establishment of overt metastatic lesions (199). Consequently, treatment with autophagy inhibitors after the development of proliferative lesions (i.e. lesions that moved beyond the dormant-to-proliferative switch) had lesser impact on the metastatic burden (199).

These observations indicate that autophagy plays an active role in the initiation and maintenance of the dormant state, as well as during the switch from dormancy to a proliferative state.

Role of Autophagy in the Clearance of Mitochondria and Regulation of Metabolism in Dormant Cancer Cells

Mitochondria are at the center of cellular energy metabolism control. A side product of oxidative phosphorylation is reactive oxygen species (ROS), and dysfunctional or damaged mitochondria are more prone to produce ROS. A life-threatening outcome of ROS accumulation at a cellular level is oxidation of building blocks such as proteins and lipids, as well as damage to DNA. A selective form of autophagy, mitophagy is a major mechanism that eliminates dysfunctional and damaged mitochondria and that ensures control of the mitochondrial mass in cells.

Cancer cells are able to stay in a dormant state for months or even years. Hence in dormant cells, in addition to elimination of unfolded/misfolded proteins and other building blocks, regulation of the mitochondrial mass and prevention of ROS accumulation should be of utmost importance for long-term survival and the preservation of reactivation capacity after transition to the proliferative state. Additionally, control of mitochondrial mass and function should be critical for metabolic reprogramming of dormant cells. Indeed, increased autophagic activity was associated with mitophagy in several models of cancer dormancy.

For instance, mitophagy was activated during DIRAS3-induced dormancy of ovarian cancer cells. Following DIRAS3 induction by the Tet-on system in ovarian cancer stable cell lines, TMRM uptake by mitochondria was decreased, indicating accumulation of depolarized mitochondria in these cells. Reduced TOM20 mitochondrial protein levels and mitochondrial mass as assessed through mitotracker staining were reported in dormant cells. As such, dormancy state was associated with a higher rate of mitochondrial depolarization, and mitophagy was increased as a mechanism to eliminate depolarized mitochondria and to limit ROS accumulation (213).

In the D2.0R breast cancer model of dormancy, autophagy protein LC3 was found to colocalize with mitochondria in cells growing in a matrix supporting dormancy. During mitophagy, PINK-assisted ubiquitylation of mitochondrial proteins by E3 ligases such as PARKIN prime mitochondria for mitophagic degradation. Indeed, accumulation of mitophagy-associated full length PINK and degradation of mitochondrial protein TOM20 was reported under these experimental conditions. Additionally, autophagy inhibition using HQ caused an accumulation of damaged mitochondria as well as ROS. Following suppression of the autophagic activity, dormant cells suffered from DNA damage, caspase-3 activation was prominent, and cells eventually died. Mitochondrial ROS scavengers prevented cell death, indicating that an important function of autophagy in dormant cells is the maintenance of healthy mitochondrial mass, hence limitation of ROS-induced damage (199). Similarly, in the TM-treated PDAC cell model of dormancy, inhibition of autophagy by CQ increased ROS accumulation and resulted in cell death, further showing that ROS limiting activity of autophagy is central to dormant cancer cell survival in different cancer models (200).

Autophagic and mitophagy activation during dormancy was associated with metabolic changes in cells. In the ovarian cancer dormancy model, induction of DIRAS3 resulted in a higher glycolytic rate and mitochondrial respiration rate was decreased (213). Indeed, ATP levels of were found to be attenuated in different models of dormancy (200, 205, 213). Moreover, in dormant cells, an increase in glucose and glutamate uptake was accompanied by extracellular lactate accumulation (213). Under these conditions, increased glucose uptake was coupled to an upregulation of glycolysis and glutaminolysis, and all these changes were autophagy dependent. In this context, blockage of these metabolic pathways resulted in decreased cell viability (213). In autophagic tumors *in vivo*, free valine, glycine, and alanine concentrations were increased at statistically significant levels, indicating that bulk degradation of proteins by autophagy was also accelerated and it further supported metabolic activities of dormant cancer cells (213).

Molecular Mechanisms of Autophagy in Dormant Cells

To date, molecular mechanisms governing autophagy activation during dormancy and autophagy signaling pathways that are involved are largely unknown. Yet, studies on autophagy-dormancy connection provided hints about the involvement of certain autophagy-related proteins and pathways in the process.

Among the upstream signaling pathways regulating autophagy, inhibition of the PI3K/AKT pathway emerges as a common observation. In many reports, mTOR pathway that is downstream to AKT was shown to be inhibited in dormant cells. As mentioned above, mTOR is a central regulator of autophagy, and its inhibition correlates with autophagy activation in various systems (214–217).

In ovarian cancer dormancy model, DIRAS3 expression resulted in the inhibition of signaling through PI3K/AKT and Ras/MAP through enhancing internalization and degradation of the epidermal growth factor receptor. As a result, mTOR signaling downstream to the AKT pathway was also inhibited (180, 201). Indeed, downregulation the pathway by DIRAS3 resulted in a decrease in the activation of mTOR downstream pathway proteins, such as p70S6K and pS6, proteins that are involved in the regulation of cell size and protein synthesis (180). Autophagic activity was strongly stimulated under these conditions.

In line with the DIRAS3 model, ascites-derived ovarian cancer spheroids were in a dormant state that was associated with AKT downregulation and autophagy activation (127, 205). In fact, inhibition of the AKT pathway in ovarian cancer cells using specific inhibitors of the AKT kinase, namely Akti-1/2, was sufficient to direct the entry of cells to a dormant-like state (127). Downregulation of AKT and mTOR pathway was also observed in the osteosarcoma dormancy model (125) and breast cancer cells entering dormancy following exposure to long-term hypoxia/reoxygenation cycles (206). Similarly, mTOR and its downstream pathways were reported to be inhibited in the imatinib-induced GIST cell dormancy model (198).

Another key protein regulating autophagy activation is the energy sensor kinase AMPK. Increased AMP/ATP ratio correlates with problems in energy status of cells, and leads to the activation of AMPK (218). Hypoxia is another signal that can activate AMPK (219). Following activation, AMPK was shown to phosphorylate TSC2 and interfere with the activity of the GTPase RHEB, an activator of mTORC1 signaling; the net result is autophagy activation (220, 221). Another mechanism through which the kinase contributes to autophagy activation direct phosphorylation of the autophagy protein ULK1 (23, 24).

In dormant cells, intracellular ATP levels are decreased (74, 205, 213). Consequently, AMPK activation was reported in different experimental models of dormancy. For instance, in ovarian tumor cells, LKB1 and AMPK expression and activity were increased during spheroid formation and dormancy (205). The study showed that LKB1 (and possibly AMPK) was required for the survival of ovarian cancer cells in a dormant state. Moreover, AMPK activation in proliferating ovarian cancer cells caused them to enter cell cycle arrest (205). Similarly, an increase in AMPK activity was observed during DIRAS-3-induced dormancy of ovarian cancer cells (180) and chronic hypoxia-induced dormancy of breast cancer cells (206).

Transcriptional upregulation of autophagy-related genes was observed in dormancy models. Independent groups reported the upregulation of key autophagy genes, including LC3, ATG4, ATG5, ATG7 and BECN1, in dormant cells (148, 199). A mechanistic explanation on the transcriptional regulation of

autophagy gene expression in the dormant state came from studies using ovarian cancer cells. During DIRAS3-induced dormancy, mTOR inhibition promoted translocation of transcription factors FOXO3a and TFEB to the nucleus (222). The end result was a FOXO3a-dependent upregulation of autophagy proteins ATG4 and LC3 and Rab7, a mediator of autophagosome-autolysosome fusion (222). Similar to FOXO3a, TFEB translocation to the nucleus activated transcription of various autophagy-related genes (223, 224).

Interestingly DIRAS3 itself was subject to transcriptional regulation downstream to the mTOR pathway (225). Under conditions of nutrient deprivation, mTOR inhibition resulted in the dissociation of E2F1 and E2F4 from the DIRAS3 promoter, leading to the proteasomal degradation of these transcription factors. Dissociation of E2F1 and E2F4 from DIRAS3 promoter allowed transcriptional upregulation of the gene and activated autophagy. On the other hand, another transcription factor, CEBPα, positively regulated DIRAS3 expression and autophagy. Hence, transcriptional loops involving DIRAS3 might contribute to further activate and sustain autophagy during nutrient deprivation and possibly during dormancy (225).

DIRAS3 was directly participating to autophagy regulating protein complexes. In fact, DIRAS3 was shown to stabilize the autophagy initiation complex consisting of VPS34 (PIK3C3), BECN1 and ATG14 (201). DIRAS3 binding to BECN1 destabilized BECN1-BCL2 inhibitory complexes, displaced BCL2 and allowed recruitment of BECN1 protein by autophagy-related VPS34 lipid kinase complex. Binding of DIRAS3 to BECN1 facilitated association of BECN1 with VPS34 and ATG14. DIRAS3 was also shown to directly bind VPS34. Altogether, DIRAS3 enhanced VPS34 lipid kinase activity that is required for autophagosome formation and autophagy activation (201). Moreover, DIRAS3-mediated stabilization of the initiation complex and subsequent autophagy activation was necessary for dormant cell survival after chemotherapy (201).

On the other hand in mice, knockdown of Atg7 but not Becn1 decreased numbers of tumors formed by dormancy-prone cells in a TGFβ-induced inflammatory background, indicating that requirement for Becn1 gene in dormancy-related autophagy and tumor cell survival might be tumor and cell type-dependent (199).

Role of Autophagic Degradation in Dormancy

Data that was presented above show that autophagic activity is prominently higher in dormant cancer cells compared to their actively proliferating counterparts. Several studies provided clues about the nature of autophagy in this setting. In addition to an increase in autophagosome numbers, autolysosome formation and autolysosomal degradation was reported to be upregulated during dormancy. Autolysosomal degradation of the selective autophagy receptor p62/SQSTM1 as well as the LC3 protein itself was reported in many studies (148, 199). Inhibition of the autolysosomal activity by chemicals, such as chloroquine and its derivatives, changed the behavior of dormant cells, influenced cell survival, dormant cell reactivation and metastatic capacity. As explained above, mitochondria are among the targets of selective autophagic degradation during dormancy. Therefore, metabolic outcomes of autophagic activity in dormant cells might be attributed to mitophagy and non-selective autophagic degradation of cellular components, including long-lived proteins. So far, the role of selective autophagy in cancer dormancy is not well studied, and there are only a few reported examples.

A dormancy-related direct target of autophagy was identified using the D2.0R model of breast cancer dormancy. In this study, Pfkfb3 (6-phosphofructo-2-kinase/fructose 2,6-biphosphatase 3) was identified as a gene that was highly expressed in metastatic cells but downregulated in dormant cancer cells (148). Pfkfb3 is a key regulator of glycolysis rate in cells, and its expression was shown to promote metastatic tumor growth. An inverse correlation between dormancy-related autophagic activity and Pfkfb3 levels was observed. So, the role of autophagy in the degradation of Pfkfb3 protein was studied in dormant cancer cells. Indeed, Pfkfb3 protein accumulated when autophagic degradation was inhibited using CQ or autophagy gene knockdown. The protein was polyubiquitylated, and in this state, it directly interacted with the ubiquitin-associated domain (UBA) of the p62 protein. Strikingly, Pfkfb3 downregulation in metastatic D2.A1 breast cancer cells prevented their growth and delayed establishment of metastatic lesions. Conversely, autophagy inhibition and Pfkfb3 upregulation correlated with reactivation of dormant of D2.0R cells. The study showed that, although proteasomal degradation also contributes to the determination of protein's half-life, selective degradation by autophagy is important in the control of Pfkfb3 protein levels in dormant cells. Hence, the study provides an example where tumor dormancy and recurrence rely on autophagic clearance of metabolic regulators (148).

Another autophagy target was identified among factors regulating EMT-MET (epithelial-mesenchymal and mesenchymal- epithelial transitions) during metastasis. SYK is a non-receptor tyrosine kinase mediating signaling events downstream to several transmembrane receptors, including the B-cell receptor (BCR). Decreased expression of SYK mRNA correlated with decreased survival in breast cancer patients (226). P-bodies are cytoplasmic foci containing mRNA, miRNA and mRNA-binding proteins, and they are involved in the regulation of mRNA half-life and translation control. During TGFβ-induced EMT, accumulation of P-bodies was observed. SYK concentrated in P-bodies, and SYK activity and autophagy was necessary for controlled clearance of P-bodies during MET and metastasis (208, 227). Hence in this system, SYK promoted removal of P-bodies through autophagy and supported activation dormant cancer cells, allowing initiation of cancer metastatic outgrowth (208).

There are possibly other direct or indirect targets of autophagy that are involved in dormancy maintenance and a dormant-to-proliferative switch. Further studies will reveal the identity of these key factors that are degraded by dormancy-associated autophagic activity.

Treatment Responses, Dormancy, and Autophagy

Current cancer treatment approaches are usually unable to result in the total elimination of disseminated cancer cells and micrometastases. They even seem to create a selective pressure on cancer cells promoting their escape from cell death by

entering to a dormant state. Since dormant cells are not actively proliferating, they are in general resistant to chemotherapy and radiotherapy approaches that mainly result in DNA damage and block cell division. A body of literature provide evidence about the role of autophagy in the treatment resistance of growing primary tumors and overt metastases (228–231). Since autophagic activity is increased in dormant cells, autophagy might be a contributing factor in the observed robustness of dormant cells when faced with anticancer insults.

A number of studies tested the contribution of autophagy to treatment resistance that is observed in dormant cells. First observation is some drugs that were utilized in order to create models of dormancy, they themselves induced autophagy upregulation in cells. For example, treatment of cancer cells with imatinib (198), farnesyltransferase inhibitors (212), AKT inhibitor (204), and adriamycin (207), resulted in the upregulation of autophagic activity. Usage of antimalarial lysomorphic inhibitors of autolysosomal activity, such as Chloroquine, Hydroxychloroquine or quinacrine, as a combination treatment along with chemotherapy agents blocked autophagy under these conditions and generally resulted in the death of dormant cells and even elimination of tumors (125, 180, 202, 207). Combination of chemotherapy with genetic approaches gave similar results as well (125, 202, 207). So, capacity to activate sustained autophagy in response to cancer therapies might be one of the critical factors favoring the selection of dormant cells. This "autophagy addiction" might be exploited for the elimination of disseminated dormant cells in patients. On the other hand, considering indications about the role of autophagy in the dormant-to-proliferative switch, inhibition of autophagy might promote reactivation of dormant cancer cells, leading them to reenter an active proliferative state that renders them again susceptible to antiproliferative cancer treatments. On the other hand, crizotinib, an ALK inhibitor was shown to further activate autophagy and trigger apoptosis of dormant ovarian cancer cells (232). Either way, all these studies underline the therapeutic potential of autophagy manipulation in the context of dormancy.

CONCLUSION

Drug resistance and cancer dormancy are the two important causes of incurable metastatic disease that results in the loss of millions of lives from cancer-related deaths every year. Autophagy emerges as an integral part of the dormancy phenomenon. Autophagy activation was observed in dormant cells originating from different types of cancers, in cancer cellular models and animal models, as well as in patient-derived cells and tissues.

Autophagic activity was shown to confer survival advantage, treatment resistance and resilience to dormant cells. An important contribution of autophagy to dormant cell survival was related to the limitation of ROS accumulation. Autophagy is an important mechanism for the elimination of depolarized mitochondria, damaged peroxisomes and other organelles, as well as cytosolic long-lived proteins that are prone to aggregate and accumulate in the cytosol when exposed to excessive oxidative damage. Protection and preservation of the genetic material from ROS damage during long-lasting non-proliferative periods that may last for months or years, such as those observed during dormancy, is also an important challenge. For cells to preserve the reactivation capacity, dormant cells should be able to limit the number and extent of mutations they accumulate during periods of cell cycle arrest. Potency and efficacy of DNA repair pathways in dormant cells is not clear and further studies are required (233–235). At this point, studies in normal stem cell quiescence might give indications about the faith of DNA in cells that reside in long-term dormant periods. These studies indicated that in quiescent cells, DNA damage burden may even be higher in older cells than younger ones, and that repair process only begins following entry to cell cycle (236). Hence, these data underline the fact that, limitation of ROS accumulation by the autophagic activity contributes significantly to the survival of dormant cells.

Autophagy seems to play a critical role in the maintenance of dormant phenotype. Autophagy-deficient cells were not able to enter or stay in a dormant state compared to controls. Mechanisms through which autophagy controls the dormant phenotype are not clear. So far, only a few dormancy-related targets of autophagy were described. Pfkfb3 protein was identified as a target of selective autophagy in dormant cells. In fact, in addition to being a regulator of glycolysis, Pfkfb3 was shown to translocate to nuclei and its product fructose 2,6-biphosphate was shown to inhibit cell cycle inhibitor $p27^{Kip}$ and activate cyclin D3, resulting in progression from the G1 phase to the S phase (237). Moreover, Pfkfb3 was also involved in the upregulation of CDK1 and Cdc25 expression promoting entry to mitosis (237). Therefore, selective targeting of key proteins involved in cell cycle by autophagy, such as Pfkfb3, may be an important function of autophagy in the entry to and maintenance of the dormant phenotype.

P-bodies were also reported as selective targets of autophagy in a dormancy context. Whether autophagic degradation control a general downregulation of P-bodies or whether it is selectively and deliberately targeting a cell cycle- and dormancy-relevant subset of mRNAs, miRNAs and/or proteins is not clear.

Characterization of a full list of selective autophagy targets during dormancy will allow a better understanding of the role and contribution of autophagic degradation to the dormant phenotype.

Mechanistic aspects of autophagy signaling during dormancy are being better understood. In fact, AKT and related growth factor pathways seem to emerge as an important regulators of autophagy activation in dormant cells. Downstream to AKT, mTOR pathway components are inhibited, resulting in the activation of autophagy proteins, including ULK1. A decline in ATP levels in dormant cells may activate the energy sensor systems LKB1 and AMPK and further inhibit the mTOR pathway through TSC2 phosphorylation and activate autophagy by phosphorylating ULK1. Deficiency of nutrients, such as amino acids, possibly contribute to inhibition of mTOR on the lysosomes (238). mTOR inhibition allows activation of factors FoxO3a and TFEB, that transcriptionally upregulate autophagy-related genes.

Although most components of the canonical autophagy pathways were reported to be involved in dormancy-related autophagy, some studies questioned the contribution of key proteins, such as BECN1. Others placed BECN1 containing complexes at the center of autophagy regulation in dormant cells. ATG5 and ATG7 were reported to be important as well. p62/SQSTM1 was degraded in several independent models of dormancy, indicating that it may be an important mediator of selective autophagy under these conditions. Whether other autophagy receptors contribute to the autophagy pathway in dormant cells need further investigation.

Inhibition of autophagy by drugs or genetic methods was reported to impede dormancy, affect cell survival, or lead cells to enter into a proliferative phase, in which cancer cells are more susceptible to be eliminated by common cancer therapeutic strategies. Hence, approaches involving promotion of dormancy or reactivation of dormant cells should both necessitate study and manipulation of autophagy. A detailed understanding of mechanisms, regulatory pathways and specific targets of autophagy in the context of dormancy will certainly contribute to a better management of metastatic and recurrent disease, and maybe allow one day total elimination of disseminated cells and micrometastases in all cancer patients.

AUTHOR CONTRIBUTIONS

DG designed the structure of the review and wrote and edited the review article. YA and NP wrote the review article and prepared the tables and figures. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by Scientific and Technological Research Council of Turkey (TUBITAK) grant number 216S489.

REFERENCES

1. Liu Q, Zhang H, Jiang X, Qian C, Liu Z, Luo D. Factors involved in cancer metastasis: A better understanding to "seed and soil" hypothesis. *Mol Cancer* (2017) 16. doi: 10.1186/s12943-017-0742-4
2. Dongre A, Weinberg RA. New insights into the mechanisms of epithelial–mesenchymal transition and implications for cancer. *Nat Rev Mol Cell Biol* (2019) 20:69–84. doi: 10.1038/s41580-018-0080-4
3. Korpal M, Lee ES, Hu G, Kang Y. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. *J Biol Chem* (2008) 283:14910–4. doi: 10.1074/jbc.C800074200
4. Nieto MA. The snail superfamily of zinc-finger transcription factors. *Nat Rev Mol Cell Biol* (2002). doi: 10.1038/nrm757
5. Carver EA, Jiang R, Lan Y, Oram KF, Gridley T. The Mouse Snail Gene Encodes a Key Regulator of the Epithelial-Mesenchymal Transition. *Mol Cell Biol* (2001) 21:8184–8. doi: 10.1128/mcb.21.23.8184-8188.2001
6. Chaffer CL, San Juan BP, Lim E, Weinberg RA. EMT, cell plasticity and metastasis. *Cancer Metastasis Rev* (2016) 35:645–54. doi: 10.1007/s10555-016-9648-7
7. Bennett DC, Peachey LA, Durbin H, Rudland PS. A possible mammary stem cell line. *Cell* (1978) 15:283–98. doi: 10.1016/0092-8674(78)90104-6
8. Hager JC, Heppner GH, Stanley W, Richardson AM, Fligiel S. Characterization of a Variant-Producing Tumor Cell Line from a Heterogeneous Strain BALB/cfC3H Mouse Mammary Tumor. *Cancer Res* (1981) 41:1293–300.
9. Schardt JA, Meyer M, Hartmann CH, Schubert F, Schmidt-Kittler O, Fuhrmann C, et al. Genomic analysis of single cytokeratin-positive cells from bone marrow reveals early mutational events in breast cancer. *Cancer Cell* (2005) 8:227–39. doi: 10.1016/j.ccr.2005.08.003
10. Hüsemann Y, Geigl JB, Schubert F, Musiani P, Meyer M, Burghart E, et al. Systemic Spread Is an Early Step in Breast Cancer. *Cancer Cell* (2008) 13:58–68. doi: 10.1016/j.ccr.2007.12.003
11. Magbanua MJM, Sosa EV, Roy R, Eisenbud LE, Scott JH, Olshen A, et al. Genomic profiling of isolated circulating tumor cells from metastatic breast cancer patients. *Cancer Res* (2013) 73:30–40. doi: 10.1158/0008-5472.CAN-11-3017
12. Pixberg CF, Raba K, Müller F, Behrens B, Honisch E, Niederacher D, et al. Analysis of DNA methylation in single circulating tumor cells. *Oncogene* (2017) 36:3223–31. doi: 10.1038/onc.2016.480
13. Lu Z, Zou J, Li S, Topper MJ, Tao Y, Zhang H, et al. Epigenetic therapy inhibits metastases by disrupting premetastatic niches. *Nature* (2020) 579:284–90. doi: 10.1038/s41586-020-2054-x
14. Holmgren L, O'reilly MS, Folkman J. Dormancy of micrometastases: Balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nat Med* (1995) 1:149–53. doi: 10.1038/nm0295-149
15. Karrison TG, Ferguson DJ, Meier P. Dormancy of mammary carcinoma after mastectomy. *J Natl Cancer Inst* (1999) 91:80–5. doi: 10.1093/jnci/91.1.80
16. Kobayashi H, Mochizuki H, Sugihara K, Morita T, Kotake K, Teramoto T, et al. Characteristics of recurrence and surveillance tools after curative resection for colorectal cancer: A multicenter study. *Surgery* (2007) 141:67–75. doi: 10.1016/j.surg.2006.07.020
17. Brookman-May SD, May M, Shariat SF, Novara G, Zigeuner R, Cindolo L, et al. Time to recurrence is a significant predictor of cancer-specific survival after recurrence in patients with recurrent renal cell carcinoma - Results from a comprehensive multi-centre database (CORONA/SATURN-Project). *BJU Int* (2013) 112:909–16. doi: 10.1111/bju.12246
18. Lee J, Kim SH, Kang BJ. Prognostic Factors of Disease Recurrence in Breast Cancer Using Quantitative and Qualitative Magnetic Resonance Imaging (MRI) Parameters. *Sci Rep* (2020) 10. doi: 10.1038/s41598-020-64564-6
19. Sosa MS, Bragado P, Debnath J, Aguirre-Ghiso JA. Regulation of tumor cell dormancy by tissue microenvironments and autophagy. *Adv Exp Med Biol* (2013) 734:73–89. doi: 10.1007/978-1-4614-1445-2_5
20. Arias E, Cuervo AM. Pros and Cons of Chaperone-Mediated Autophagy in Cancer Biology. *Trends Endocrinol Metab* (2020) 31:53–66. doi: 10.1016/j.tem.2019.09.007
21. Jung CH, Ro SH, Cao J, Otto NM, Kim DH. MTOR regulation of autophagy. *FEBS Lett* (2010) 584:1287–95. doi: 10.1016/j.febslet.2010.01.017
22. Zoncu R, Efeyan A, Sabatini DM. MTOR: From growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* (2011). doi: 10.1038/nrm3025
23. Egan DF, Shackelford DB, Mihaylova MM, Gelino S, Kohnz RA, Mair W, et al. Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. *Science (80-)* (2011) 331:456–61. doi: 10.1126/science.1196371
24. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol* (2011) 13:132–41. doi: 10.1038/ncb2152
25. Tripathi DN, Chowdhury R, Trudel LJ, Tee AR, Slack RS, Walker CL, et al. Reactive nitrogen species regulate autophagy through ATM-AMPK-TSC2-mediated suppression of mTORC1. *Proc Natl Acad Sci USA* (2013) 110. doi: 10.1073/pnas.1307736110
26. Bar-Peled L, Sabatini DM. Regulation of mTORC1 by amino acids. *Trends Cell Biol* (2014) 24:400–6. doi: 10.1016/j.tcb.2014.03.003

27. Dikic I, Elazar Z. Mechanism and medical implications of mammalian autophagy. *Nat Rev Mol Cell Biol* (2018) 19:349–64. doi: 10.1038/s41580-018-0003-4
28. Ozturk DG, Kocak M, Akcay A, Kinoglu K, Kara E, Buyuk Y, et al. MITF-MIR211 axis is a novel autophagy amplifier system during cellular stress. *Autophagy* (2019) 15:375–90. doi: 10.1080/15548627.2018.1531197
29. Hosokawa N, Hara T, Kaizuka T, Kishi C, Takamura A, Miura Y, et al. Nutrient-dependent mTORCl association with the ULK1-Atg13-FIP200 complex required for autophagy. *Mol Biol Cell* (2009) 20:1981–91. doi: 10.1091/mbc.E08-12-1248
30. Jung CH, Jun CB, Ro SH, Kim YM, Otto NM, Cao J, et al. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol Biol Cell* (2009) 20:1992–2003. doi: 10.1091/mbc.E08-12-1249
31. Di Bartolomeo S, Corazzari M, Nazio F, Oliverio S, Lisi G, Antonioli M, et al. The dynamic interaction of AMBRA1 with the dynein motor complex regulates mammalian autophagy. *J Cell Biol* (2010) 191:155–68. doi: 10.1083/jcb.201002100
32. Karanasios E, Stapleton E, Manifava M, Kaizuka T, Mizushima N, Walker SA, et al. Dynamic association of the ULK1 complex with omegasomes during autophagy induction. *J Cell Sci* (2013) 126:5224–38. doi: 10.1242/jcs.132415
33. Hamasaki M, Furuta N, Matsuda A, Nezu A, Yamamoto A, Fujita N, et al. Autophagosomes form at ER-mitochondria contact sites. *Nature* (2013) 495:389–93. doi: 10.1038/nature11910
34. Polson HEJ, De Lartigue J, Rigden DJ, Reedijk M, Urbé S, Clague MJ, et al. Mammalian Atg18 (WIPI2) localizes to omegasome-anchored phagophores and positively regulates LC3 lipidation. *Autophagy* (2010) 6:506–22. doi: 10.4161/auto.6.4.11863
35. Dooley HC, Razi M, Polson HEJ, Girardin SE, Wilson MI, Tooze SA. WIPI2 Links LC3 Conjugation with PI3P, Autophagosome Formation, and Pathogen Clearance by Recruiting Atg12-5-16L1. *Mol Cell* (2014) 55:238–52. doi: 10.1016/j.molcel.2014.05.021
36. Axe EL, Walker SA, Manifava M, Chandra P, Roderick HL, Habermann A, et al. Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. *J Cell Biol* (2008) 182:685–701. doi: 10.1083/jcb.200803137
37. Rawet Slobodkin M, Elazar Z. The Atg8 family: Multifunctional ubiquitin-like key regulators of autophagy. *Essays Biochem* (2013) 55:51–64. doi: 10.1042/BSE0550051
38. Noda NN, Ohsumi Y, Inagaki F. Atg8-family interacting motif crucial for selective autophagy. *FEBS Lett* (2010) 584:1379–85. doi: 10.1016/j.febslet.2010.01.018
39. Ichimura Y, Kumanomidou T, Sou YS, Mizushima T, Ezaki J, Ueno T, et al. Structural basis for sorting mechanism of p62 in selective autophagy. *J Biol Chem* (2008) 283:22847–57. doi: 10.1074/jbc.M802182200
40. Yoshii SR, Mizushima N. Monitoring and measuring autophagy. *Int J Mol Sci* (2017) 18. doi: 10.3390/ijms18091865
41. Mizushima N, Yoshimori T, Levine B. Methods in Mammalian Autophagy Research. *Cell* (2010) 140:313–26. doi: 10.1016/j.cell.2010.01.028
42. Liu R, Zhi X, Zhong Q. ATG14 controls SNARE-mediated autophagosome fusion with a lysosome. *Autophagy* (2015) 11:847–9. doi: 10.1080/15548627.2015.1037549
43. Peker N, Gozuacik D. Autophagy as a Cellular Stress Response Mechanism in the Nervous System. *J Mol Biol* (2020) 432:2560–88. doi: 10.1016/j.jmb.2020.01.017
44. Bayraktar O, Oral O, Kocaturk NM, Akkoc Y, Eberhart K, Kosar A, et al. IBMPFD disease-causing mutant VCP/p97 proteins are targets of autophagic-lysosomal degradation. *PloS One* (2016) 11. doi: 10.1371/journal.pone.0164864
45. Gozuacik D, Akkoc Y, Gulfem Ozturk D, Kocak M. Autophagy-Regulating microRNAs and Cancer. *Front Oncol* (2017) 7. doi: 10.3389/fonc.2017.00065
46. Karakaş HE, Gözüaçik D. Autophagy and cancer. *Turkish J Biol* (2014) 38:720–39. doi: 10.3906/biy-1408-16
47. Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G, Chen HY, et al. Autophagy Suppresses Tumorigenesis through Elimination of p62. *Cell* (2009) 137:1062–75. doi: 10.1016/j.cell.2009.03.048
48. Levine B. Autophagy and cancer. *Nature* (2007) 446:745–7. doi: 10.1038/446745a
49. Fernandez Larrosa PN, Alvarado CV, Rubio MF, Ruiz Grecco M, Micenmacher S, Martinez-Noel GA, et al. Nuclear receptor coactivator RAC3 inhibits autophagy. *Cancer Sci* (2012) 103:2064–71. doi: 10.1111/cas.12019
50. Kocaturk NM, Akkoc Y, Kig C, Bayraktar O, Gozuacik D, Kutlu O. Autophagy as a molecular target for cancer treatment. *Eur J Pharm Sci* (2019) 134:116–37. doi: 10.1016/j.ejps.2019.04.011
51. Unal O, Akkoc Y, Kocak M, Nalbat E, Dogan-Ekici AI, Yagci Acar H, et al. Treatment of breast cancer with autophagy inhibitory microRNAs carried by AGO2-conjugated nanoparticles. *J Nanobiotechnol* (2020) 18. doi: 10.1186/s12951-020-00615-4
52. Mathiassen SG, De Zio D, Cecconi F. Autophagy and the cell cycle: A complex landscape. *Front Oncol* (2017) 7. doi: 10.3389/fonc.2017.00051
53. Vlahakis A, Debnath J. The Interconnections between Autophagy and Integrin-Mediated Cell Adhesion. *J Mol Biol* (2017) 429:515–30. doi: 10.1016/j.jmb.2016.11.027
54. Gerada C, Ryan KM. Autophagy, the innate immune response and cancer. *Mol Oncol* (2020) 14:1913–29. doi: 10.1002/1878-0261.12774
55. Gozuacik D, Kimchi A. Autophagy as a cell death and tumor suppressor mechanism. *Oncogene* (2004) 23:2891–906. doi: 10.1038/sj.onc.1207521
56. Morselli E, Galluzzi L, Kepp O, Vicencio JM, Criollo A, Maiuri MC, et al. Anti- and pro-tumor functions of autophagy. *Biochim Biophys Acta - Mol Cell Res* (2009) 1793:1524–32. doi: 10.1016/j.bbamcr.2009.01.006
57. Hadfield G. The dormant cancer cell. *Obstet Gynecol Surv* (1955) 10:916–9. doi: 10.1097/00006254-195512000-00045
58. Sosa MS, Bragado P, Aguirre-Ghiso JA. Mechanisms of disseminated cancer cell dormancy: An awakening field. *Nat Rev Cancer* (2014) 14:611–22. doi: 10.1038/nrc3793
59. Yeh AC, Ramaswamy S. Mechanisms of cancer cell dormancy-another hallmark of cancer? *Cancer Res* (2015) 75:5014–22. doi: 10.1158/0008-5472.CAN-15-1370
60. Spiliotaki M, Mavroudis D, Kapranou K, Markomanolaki H, Kallergi G, Koinis F, et al. Evaluation of proliferation and apoptosis markers in circulating tumor cells of women with early breast cancer who are candidates for tumor dormancy. *Breast Cancer Res* (2014) 16. doi: 10.1186/s13058-014-0485-8
61. Marlow R, Honeth G, Lombardi S, Cariati M, Hessey S, Pipili A, et al. A novel model of dormancy for bone metastatic breast cancer cells. *Cancer Res* (2013) 73:6886–99. doi: 10.1158/0008-5472.CAN-13-0991
62. Darzynkiewicz Z, Juan G, Srour EF. Differential staining of DNA and RNA. *Curr Protoc Cytom* (2004). doi: 10.1002/0471142956.cy0703s30
63. Zou P, Yoshihara H, Hosokawa K, Tai I, Shinmyozu K, Tsukahara F, et al. P57 Kip2 and p27 Kip1 cooperate to maintain hematopoietic stem cell quiescence through interactions with Hsc70. *Cell Stem Cell* (2011) 9:247–61. doi: 10.1016/j.stem.2011.07.003
64. Lwin T, Hazlehurst LA, Dessureault S, Lai R, Bai W, Sotomayor E, et al. Cell adhesion induces p27Kip1-associated cell-cycle arrest through down-regulation of the SCFSkp2 ubiquitin ligase pathway in mantle-cell and other non-Hodgkin B-cell lymphomas. *Blood* (2007) 110:1631–8. doi: 10.1182/blood-2006-11-060350
65. Sadasivam S, DeCaprio JA. The DREAM complex: Master coordinator of cell cycle-dependent gene expression. *Nat Rev Cancer* (2013) 13:585–95. doi: 10.1038/nrc3556
66. Schmit F, Korenjak M, Mannefeld M, Schmitt K, Franke C, Von Eyss B, et al. LINC, a human complex that is related to pRB-containing complexes in invertebrates regulates the expression of G2/M genes. *Cell Cycle* (2007) 6:1903–13. doi: 10.4161/cc.6.15.4512
67. Litovchick L, Florens LA, Swanson SK, Washburn MP, Decaprio JA. DYRK1A protein kinase promotes quiescence and senescence through DREAM complex assembly. *Genes Dev* (2011) 25:801–13. doi: 10.1101/gad.2034211
68. Besson A, Gurian-West M, Chen X, Kelly-Spratt KS, Kemp CJ, Roberts JM. A pathway in quiescent cells that controls p27Kip1 stability, subcellular localization, and tumor suppression. *Genes Dev* (2006) 20:47–64. doi: 10.1101/gad.1384406
69. Deng X, Ewton DZ, Friedman E. Mirk dyrk1B maintains the viability of quiescent pancreatic cancer cells by reducing levels of reactive oxygen species. *Cancer Res* (2009) 69:3317–24. doi: 10.1158/0008-5472.CAN-08-2903

70. Aguirre-Ghiso JA, Estrada Y, Liu D, Ossowski L. ERKMAPK activity as a determinant of tumor growth and dormancy; regulation by p38SAPK. *Cancer Res* (2003) 63:1684–95. doi: 10.1016/j.urolonc.2003.12.012
71. Khaled AR, Bulavin DV, Kittipatarin C, Li WQ, Alvarez M, Kim K, et al. Cytokine-driven cell cycling is mediated through Cdc25A. *J Cell Biol* (2005) 169:755–63. doi: 10.1083/jcb.200409099
72. Adam AP, George A, Schewe D, Bragado P, Iglesias BV, Ranganathan AC, et al. Computational identification of a p38 SAPK-regulated transcription factor network required for tumor cell quiescence. *Cancer Res* (2009) 69:5664–72. doi: 10.1158/0008-5472.CAN-08-3820
73. Bragado P, Estrada Y, Parikh F, Krause S, Capobianco C, Farina HG, et al. TGF-β2 dictates disseminated tumour cell fate in target organs through TGF-β-RIII and p38α/β signalling. *Nat Cell Biol* (2013) 15:1351–61. doi: 10.1038/ncb2861
74. Yu-Lee LY, Yu G, Lee YC, Lin SC, Pan J, Pan T, et al. Osteoblast-secreted factors mediate dormancy of metastatic prostate cancer in the bone via activation of the TGFbRIII–p38MAPK–pS249/ T252RB pathway. *Cancer Res* (2018) 78:2911–24. doi: 10.1158/0008-5472.CAN-17-1051
75. Yumoto K, Eber MR, Wang J, Cackowski FC, Decker AM, Lee E, et al. Axl is required for TGF-β2-induced dormancy of prostate cancer cells in the bone marrow. *Sci Rep* (2016) 6. doi: 10.1038/srep36520
76. Aguirre-Ghiso JA, Liu D, Mignatti A, Kovalski K, Ossowski L. Urokinase receptor and fibronectin regulate the ERKMAPK to p38MAPK activity ratios that determine carcinoma cell proliferation or dormancy in vivo. *Mol Biol Cell* (2001) 12:863–79. doi: 10.1091/mbc.12.4.863
77. Litchfield LM, Riggs KA, Hockenberry AM, Oliver LD, Barnhart KG, Cai J, et al. Identification and characterization of nucleolin as a COUP-TFII coactivator of retinoic acid receptor β transcription in breast cancer cells. *PloS One* (2012) 7. doi: 10.1371/journal.pone.0038278
78. Aguirre Ghiso JA. Inhibition of FAK signaling activated by urokinase receptor induces dormancy in human carcinoma cells in vivo. *Oncogene* (2002) 21:2513–24. doi: 10.1038/sj.onc.1205342
79. Gao XL, Zheng M, Wang HF, Dai LL, Yu XH, Yang X, et al. NR2F1 contributes to cancer cell dormancy, invasion and metastasis of salivary adenoid cystic carcinoma by activating CXCL12/CXCR4 pathway. *BMC Cancer* (2019) 19. doi: 10.1186/s12885-019-5925-5
80. Zhang XHF, Wang Q, Gerald W, Hudis CA, Norton L, Smid M, et al. Latent Bone Metastasis in Breast Cancer Tied to Src-Dependent Survival Signals. *Cancer Cell* (2009) 16:67–78. doi: 10.1016/j.ccr.2009.05.017
81. Kobayashi A, Okuda H, Xing F, Pandey PR, Watabe M, Hirota S, et al. Bone morphogenetic protein 7 in dormancy and metastasis of prostate cancer stem-like cells in bone. *J Exp Med* (2011) 208:2641–55. doi: 10.1084/jem.20110840
82. Sharma S, Xing F, Liu Y, Wu K, Said N, Pochampally R, et al. Secreted protein acidic and rich in cysteine (sparc) mediates metastatic dormancy of prostate cancer in bone. *J Biol Chem* (2016) 291:19351–63. doi: 10.1074/jbc.M116.737379
83. Holtzhausen A, Golzio C, How T, Lee YH, Schiemann WP, Katsanis N, et al. Novel bone morphogenetic protein signaling through Smad2 and Smad3 to regulate cancer progression and development. *FASEB J* (2014) 28:1248–67. doi: 10.1096/fj.13-239178
84. Gao H, Chakraborty G, Lee-Lim AP, Mo Q, Decker M, Vonica A, et al. The BMP inhibitor Coco reactivates breast cancer cells at lung metastatic sites. *Cell* (2012) 150:764–79. doi: 10.1016/j.cell.2012.06.035
85. Kim RS, Avivar-Valderas A, Estrada Y, Bragado P, Sosa MS, Aguirre-Ghiso JA, et al. Dormancy signatures and metastasis in estrogen receptor positive and negative breast cancer. *PloS One* (2012) 7. doi: 10.1371/journal.pone.0035569
86. McGrath J, Panzica L, Ransom R, Withers HG, Gelman IH. Identification of genes regulating breast cancer dormancy in 3D bone endosteal niche cultures. *Mol Cancer Res* (2019) 17:860–9. doi: 10.1158/1541-7786.MCR-18-0956
87. Sosa MS, Parikh F, Maia AG, Estrada Y, Bosch A, Bragado P, et al. NR2F1 controls tumour cell dormancy via SOX9- and RARβ-driven quiescence programmes. *Nat Commun* (2015) 7. doi: 10.1038/ncomms7170
88. Shiozawa Y, Pedersen EA, Patel LR, Ziegler AM, Havens AM, Jung Y, et al. GAS6/AXL axis regulates prostate cancer invasion, proliferation, and survival in the bone marrow niche. *Neoplasia* (2010) 12:116–27. doi: 10.1593/neo.91384
89. Taichman RS, Patel LR, Bedenis R, Wang J, Weidner S, Schumann T, et al. GAS6 Receptor Status Is Associated with Dormancy and Bone Metastatic Tumor Formation. *PloS One* (2013) 8. doi: 10.1371/journal.pone.0061873
90. Shiozawa Y, Pedersen EA, Taichman RS. GAS6/Mer axis regulates the homing and survival of the E2A/PBX1-positive B-cell precursor acute lymphoblastic leukemia in the bone marrow niche. *Exp Hematol* (2010) 38:132–40. doi: 10.1016/j.exphem.2009.11.002
91. Khoo WH, Ledergor G, Weiner A, Roden DL, Terry RL, McDonald MM, et al. A niche-dependent myeloid transcriptome signature defines dormant myeloma cells. *Blood* (2019) 134:30–43. doi: 10.1182/blood.2018880930
92. Harper KL, Sosa MS, Entenberg D, Hosseini H, Cheung JF, Nobre R, et al. Mechanism of early dissemination and metastasis in Her2+ mammary cancer. *Nature* (2016) 540:588–92. doi: 10.1038/nature20609
93. Buczacki SJA, Popova S, Biggs E, Koukorava C, Buzzelli J, Vermeulen L, et al. Itraconazole targets cell cycle heterogeneity in colorectal cancer. *J Exp Med* (2018) 215:1891–912. doi: 10.1084/jem.20171385
94. Ren D, Dai Y, Yang Q, Zhang X, Guo W, Ye L, et al. Wnt5a induces and maintains prostate cancer cells dormancy in bone. *J Exp Med* (2019) 216:428–49. doi: 10.1084/jem.20180661
95. Malladi S, MacAlinao DG, Jin X, He L, Basnet H, Zou Y, et al. Metastatic Latency and Immune Evasion through Autocrine Inhibition of WNT. *Cell* (2016) 165:45–60. doi: 10.1016/j.cell.2016.02.025
96. Boyerinas B, Zafrir M, Yesilkanal AE, Price TT, Hyjek EM, Sipkins DA. Adhesion to osteopontin in the bone marrow niche regulates lymphoblastic leukemia cell dormancy. *Blood* (2013) 121:4821–31. doi: 10.1182/blood-2012-12-475483
97. Yu N, Seedhouse C, Russell N, Pallis M. Quantitative assessment of the sensitivity of dormant AML cells to the BAD mimetics ABT-199 and ABT-737. *Leuk Lymphoma* (2018) 59:2447–53. doi: 10.1080/10428194.2018.1434884
98. Najmi S, Korah R, Chandra R, Abdellatif M, Wieder R. Flavopiridol blocks integrin-mediated survival in dormant breast cancer cells. *Clin Cancer Res* (2005) 11:2038–46. doi: 10.1158/1078-0432.CCR-04-1083
99. Bambang IF, Lu D, Li H, Chiu LL, Lau QC, Koay E, et al. Cytokeratin 19 regulates endoplasmic reticulum stress and inhibits ERp29 expression via p38 MAPK/XBP-1 signaling in breast cancer cells. *Exp Cell Res* (2009) 315:1964–74. doi: 10.1016/j.yexcr.2009.02.017
100. Amin R, Morita-Fujimura Y, Tawarayama H, Semba K, Chiba N, Fukumoto M, et al. δNp63α induces quiescence and downregulates the BRCA1 pathway in estrogen receptor-positive luminal breast cancer cell line MCF7 but not in other breast cancer cell lines. *Mol Oncol* (2016) 10:575–93. doi: 10.1016/j.molonc.2015.11.009
101. Sanchez Calle A, Yamamoto T, Kawamura Y, Hironaka-Mitsuhashi A, Ono M, Tsuda H, et al. Long non-coding NR2F1-AS1 is associated with tumor recurrence in estrogen receptor-positive breast cancers. *Mol Oncol* (2020) 14:2271–87. doi: 10.1002/1878-0261.12704
102. Wesley Overton K, Spencer SL, Noderer WL, Meyer T, Wang CL. Basal p21 controls population heterogeneity in cycling and quiescent cell cycle states. *Proc Natl Acad Sci USA* (2014) 111:E4386–93. doi: 10.1073/pnas.1409797111
103. Bliss SA, Sinha G, Sandiford OA, Williams LM, Engelberth DJ, Guiro K, et al. Mesenchymal stem cell-derived exosomes stimulate cycling quiescence and early breast cancer dormancy in bone marrow. *Cancer Res* (2016) 76:5832–44. doi: 10.1158/0008-5472.CAN-16-1092
104. El-Shennawy L, Dubrovskyi O, Kastrati I, Danes JM, Zhang Y, Whiteley HE, et al. Coactivation of estrogen receptor and IKKb induces a dormant metastatic phenotype in ER-Positive breast cancer. *Cancer Res* (2018) 78:974–84. doi: 10.1158/0008-5472.CAN-17-1686
105. Huang J, Woods P, Normolle D, Goff JP, Benos PV, Stehle CJ, et al. Downregulation of estrogen receptor and modulation of growth of breast cancer cell lines mediated by paracrine stromal cell signals. *Breast Cancer Res Treat* (2017) 161:229–43. doi: 10.1007/s10549-016-4052-0
106. Lu D, Chen S, Tan X, Li N, Liu C, Li Z, et al. Fra-1 promotes breast cancer chemosensitivity by driving cancer stem cells from dormancy. *Cancer Res* (2012) 72:3451–6. doi: 10.1158/0008-5472.CAN-11-2536
107. Weidenfeld K, Schif-Zuck S, Abu-Tayeh H, Kang K, Kessler O, Weissmann M, et al. Dormant tumor cells expressing LOXL2 acquire a stem-like phenotype mediating their transition to proliferative growth. *Oncotarget* (2016) 7:71362–77. doi: 10.18632/oncotarget.12109

108. Barkan D, Kleinman H, Simmons JL, Asmussen H, Kamaraju AK, Hoenorhoff MJ, et al. Inhibition of metastatic outgrowth from single dormant tumor cells by targeting the cytoskeleton. *Cancer Res* (2008) 68:6241–50. doi: 10.1158/0008-5472.CAN-07-6849
109. Touny LHE, Vieira A, Mendoza A, Khanna C, Hoenerhoff MJ, Green JE. Combined SFK/MEK inhibition prevents metastatic outgrowth of dormant tumor cells. *J Clin Invest* (2014) 124:156–68. doi: 10.1172/JCI70259
110. Chakraborty S, Jiang C, Gau D, Oddo M, Ding Z, Vollmer L, et al. Profilin-1 deficiency leads to SMAD3 upregulation and impaired 3D outgrowth of breast cancer cells. *Br J Cancer* (2018) 119:1106–17. doi: 10.1038/s41416-018-0284-6
111. Johnson RW, Sun Y, Ho PWM, Chan ASM, Johnson JA, Pavlos NJ, et al. Parathyroid hormone-related protein negatively regulates tumor cell dormancy genes in a PTHR1/cyclic AMP-independent manner. *Front Endocrinol (Lausanne)* (2018) 9:241. doi: 10.3389/fendo.2018.00241
112. Romero-Moreno R, Curtis KJ, Coughlin TR, Cristina Miranda-Vergara M, Dutta S, Natarajan A, et al. The CXCL5/CXCR2 axis is sufficient to promote breast cancer colonization during bone metastasis. *Nat Commun* (2019) 10. doi: 10.1038/s41467-019-12108-6
113. Chatterjee M, Van Golen KL. Farnesyl transferase inhibitor treatment of breast cancer cells leads to altered RhoA and RhoC GTPase activity and induces a dormant phenotype. *Int J Cancer* (2011) 129:61–9. doi: 10.1002/ijc.25655
114. De Cock JM, Shibue T, Dongre A, Keckesova Z, Reinhardt F, Weinberg RA. Inflammation triggers Zeb1-dependent escape from tumor latency. *Cancer Res* (2016) 76:6778–84. doi: 10.1158/0008-5472.CAN-16-0608
115. Francescangeli F, Contavalli P, De Angelis ML, Careccia S, Signore M, Haas TL, et al. A pre-existing population of ZEB2+ quiescent cells with stemness and mesenchymal features dictate chemoresistance in colorectal cancer. *J Exp Clin Cancer Res* (2020) 39:1–17. doi: 10.1186/s13046-019-1505-4
116. Heckmann D, Maier P, Laufs S, Wenz F, Zeller WJ, Fruehauf S, et al. CXCR4 expression and treatment with SDF-1α or plerixafor modulate proliferation and chemosensitivity of colon cancer cells. *Transl Oncol* (2013) 6:124–32. doi: 10.1593/tlo.12268
117. Zhou Y, Su Y, Zhu H, Wang X, Li X, Dai C, et al. Interleukin-23 receptor signaling mediates cancer dormancy and radioresistance in human esophageal squamous carcinoma cells via the Wnt/Notch pathway. *J Mol Med* (2019) 97:177–88. doi: 10.1007/s00109-018-1724-8
118. Adamski V, Hattermann K, Kubelt C, Cohrs G, Lucius R, Synowitz M, et al. Entry and exit of chemotherapeutically-promoted cellular dormancy in glioblastoma cells is differentially affected by the chemokines CXCL12, CXCL16, and CX3CL1. *Oncogene* (2020) 39:4421–35. doi: 10.1038/s41388-020-1302-8
119. Hofstetter CP, Burkhardt JK, Shin BJ, Gürsel DB, Mubita L, Gorrepati R, et al. Protein phosphatase 2A mediates dormancy of glioblastoma multiforme-derived tumor stem-like cells during hypoxia. *PloS One* (2012) 7. doi: 10.1371/journal.pone.0030059
120. Yang LY, Shan YM, Zhang Y, Zhou EH, Chen XP, Zhang H. Aurora kinase A induces chemotherapy resistance through revival of dormant cells in laryngeal squamous cell carcinoma. *Head Neck* (2019) 41:2239–48. doi: 10.1002/hed.25689
121. Boix L, López-Oliva JM, Rhodes AC, Bruix J. Restoring miR122 in human stem-like hepatocarcinoma cells, prompts tumor dormancy through Smad-independent TGF-ß pathway. *Oncotarget* (2016) 7:71309–29. doi: 10.18632/oncotarget.11885
122. Dai Y, Wang L, Tang J, Cao P, Luo Z, Sun J, et al. Activation of anaphase-promoting complex by p53 induces a state of dormancy in cancer cells against chemotherapeutic stress. *Oncotarget* (2016) 7:25478–92. doi: 10.18632/oncotarget.8172
123. Liu Y, Lv J, Liang X, Yin X, Zhang L, Chen D, et al. Fibrin stiffness mediates dormancy of tumor-repopulating cells via a Cdc42-driven Tet2 epigenetic program. *Cancer Res* (2018) 78:3926–37. doi: 10.1158/0008-5472.CAN-17-3719
124. Flores-Guzmán F, Utikal J, Umansky V. Dormant tumor cells interact with memory CD8+ T cells in RET transgenic mouse melanoma model. *Cancer Lett* (2020) 474:74–81. doi: 10.1016/j.canlet.2020.01.016
125. Shimizu T, Sugihara E, Yamaguchi-Iwai S, Tamaki S, Koyama Y, Kamel W, et al. IGF2 preserves osteosarcoma cell survival by creating an autophagic state of dormancy that protects cells against chemotherapeutic stress. *Cancer Res* (2014) 74:6531–41. doi: 10.1158/0008-5472.CAN-14-0914
126. MacDonald J, Ramos-Valdes Y, Perampalam P, Litovchick L, DiMattia GE, Dick FA. A systematic analysis of negative growth control implicates the DREAM complex in cancer cell dormancy. *Mol Cancer Res* (2017) 15:371–81. doi: 10.1158/1541-7786.MCR-16-0323-T
127. Correa RJM, Peart T, Valdes YR, Dimattia GE, Shepherd TG. Modulation of AKT activity is associated with reversible dormancy in ascites-derived epithelial ovarian cancer spheroids. *Carcinogenesis* (2012) 33:49–58. doi: 10.1093/carcin/bgr241
128. Kinoshita Y, Kalir T, Rahaman J, Dottino P, Kohtz DS. Alterations in nuclear pore architecture allow cancer cell entry into or exit from drug-resistant dormancy. *Am J Pathol* (2012) 180:375–89. doi: 10.1016/j.ajpath.2011.09.024
129. Cheng J, Cashman JR. PAWI-2 overcomes tumor stemness and drug resistance via cell cycle arrest in integrin β3-KRAS-dependent pancreatic cancer stem cells. *Sci Rep* (2020) 10:1–11. doi: 10.1038/s41598-020-65804-5
130. Endo H, Okuyama H, Ohue M, Inoue M. Dormancy of cancer cells with suppression of AKT activity contributes to survival in chronic hypoxia. *PloS One* (2014) 9:1–12. doi: 10.1371/journal.pone.0098858
131. Owen KL, Gearing LJ, Zanker DJ, Brockwell NK, Khoo WH, Roden DL, et al. Prostate cancer cell-intrinsic interferon signaling regulates dormancy and metastatic outgrowth in bone. *EMBO Rep* (2020) 21. doi: 10.15252/embr.202050162
132. Ruppender N, Larson S, Lakely B, Kollath L, Brown L, Coleman I, et al. Cellular adhesion promotes prostate cancer cells escape from dormancy. *PloS One* (2015) 10. doi: 10.1371/journal.pone.0130565
133. Indraccolo S, Minuzzo S, Masiero M, Pusceddu I, Persano L, Moserle L, et al. Cross-talk between tumor and endothelial cells involving the Notch3-DII4 interaction marks escape from tumor dormancy. *Cancer Res* (2009) 69:1314–23. doi: 10.1158/0008-5472.CAN-08-2791
134. Saudemont A, Jouy N, Hetuin D, Quesnel B. NK cells that are activated by CXCL10 can kill dormant tumor cells that resist CTL-mediated lysis and can express B7-H1 that stimulates T cells. *Blood* (2005) 105:2428–35. doi: 10.1182/blood-2004-09-3458
135. Hurst RE, Hauser PJ, Kyker KD, Heinlen JE, Hodde JP, Hiles MC, et al. Suppression and Activation of the Malignant Phenotype by Extracellular Matrix in Xenograft Models of Bladder Cancer: A Model for Tumor Cell "Dormancy." *PloS One* (2013) 8. doi: 10.1371/journal.pone.0064181
136. Arlt MJE, Banke IJ, Bertz J, Ram Kumar RM, Muff R, Born W, et al. Reduced latency in the metastatic niche contributes to the more aggressive phenotype of LM8 compared to dunn osteosarcoma cells. *Sarcoma* (2013) 2013. doi: 10.1155/2013/404962
137. Watson KL, Jones RA, Bruce A, Moorehead RA. The miR-200b/200a/429 cluster prevents metastasis and induces dormancy in a murine claudin-low mammary tumor cell line. *Exp Cell Res* (2018) 369:17–26. doi: 10.1016/j.yexcr.2018.04.024
138. Ghajar CM, Peinado H, Mori H, Matei IR, Evason KJ, Brazier H, et al. The perivascular niche regulates breast tumour dormancy. *Nat Cell Biol* (2013) 15:807–17. doi: 10.1038/ncb2767
139. Gawrzak S, Rinaldi L, Gregorio S, Arenas EJ, Salvador F, Urosevic J, et al. MSK1 regulates luminal cell differentiation and metastatic dormancy in ER + breast cancer. *Nat Cell Biol* (2018) 20:211–21. doi: 10.1038/s41556-017-0021-z
140. Marshall JCA, Collins JW, Nakayama J, Horak CE, Liewehr DJ, Steinberg SM, et al. Effect of inhibition of the lysophosphatidic acid receptor 1 on metastasis and metastatic dormancy in breast cancer. *J Natl Cancer Inst* (2012) 104:1306–19. doi: 10.1093/jnci/djs319
141. Gattelli A, Cirio MC, Quaglino A, Schere-Levy C, Martinez N, Binaghi M, et al. Progression of pregnancy-dependent mouse mammary tumors after long dormancy periods. Involvement of Wnt pathway activation. *Cancer Res* (2004) 64:5193–9. doi: 10.1158/0008-5472.CAN-03-3992
142. Capulli M, Hristova D, Valbret Z, Carys K, Arjan R, Maurizi A, et al. Notch2 pathway mediates breast cancer cellular dormancy and mobilisation in bone and contributes to haematopoietic stem cell mimicry. *Br J Cancer* (2019) 121:157–71. doi: 10.1038/s41416-019-0501-y
143. Drescher F, Juárez P, Arellano DL, Serafín-Higuera N, Olvera-Rodriguez F, Jiménez S, et al. TIE2 induces breast cancer cell dormancy and inhibits the development of osteolytic bone metastases. *Cancers (Basel)* (2020) 12. doi: 10.3390/cancers12040868

144. Nobutani K, Shimono Y, Mizutani K, Ueda Y, Suzuki T, Kitayama M, et al. Downregulation of CXCR4 in metastasized breast cancer cells and implication in their dormancy. *PloS One* (2015) 10. doi: 10.1371/journal.pone.0130032
145. Eyob H, Ekiz HA, DeRose YS, Waltz SE, Williams MA, Welm AL. Inhibition of Ron kinase blocks conversion of micrometastases to overt metastases by boosting antitumor immunity. *Cancer Discovery* (2013) 3:751–60. doi: 10.1158/2159-8290.CD-12-0480
146. Shimizu H, Takeishi S, Nakatsumi H, Nakayama KI. Prevention of cancer dormancy by Fbxw7 ablation eradicates disseminated tumor cells. *JCI Insight* (2019) 4. doi: 10.1172/jci.insight.125138
147. Straume O, Shimamura T, Lampa MJG, Carretero J, AM Ø, Jia D, et al. Suppression of heat shock protein 27 induces long-term dormancy in human breast cancer. *Proc Natl Acad Sci USA* (2012) 109:8699–704. doi: 10.1073/pnas.1017909109
148. La Belle Flynn A, Calhoun BC, Sharma A, Chang JC, Almasan A, Schiemann WP. Autophagy inhibition elicits emergence from metastatic dormancy by inducing and stabilizing Pfkfb3 expression. *Nat Commun* (2019) 10. doi: 10.1038/s41467-019-11640-9
149. Holdman XB, Welte T, Rajapakshe K, Pond A, Coarfa C, Mo Q, et al. Upregulation of EGFR signaling is correlated with tumor stroma remodeling and tumor recurrence in FGFR1-driven breast cancer. *Breast Cancer Res* (2015) 17:1–17. doi: 10.1186/s13058-015-0649-1
150. Abravanel DL, Belka GK, Pan TC, Pant DK, Collins MA, Sterner CJ, et al. Notch promotes recurrence of dormant tumor cells following HER2/neu-targeted therapy. *J Clin Invest* (2015) 125:2484–96. doi: 10.1172/JCI74883
151. Vallabhaneni KC, Penfornis P, Xing F, Hassler Y, Adams KV, Mo YY, et al. Stromal cell extracellular Vesicular cargo mediated regulation of Breast Cancer cell Metastasis Via Ubiquitin conjugating Enzyme E2 N pathway. *Oncotarget* (2017) 8:109861–76. doi: 10.18632/oncotarget.22371
152. Bartosh TJ, Ullah M, Zeitouni S, Beaver J, Prockop DJ. Cancer cells enter dormancy after cannibalizing mesenchymal stem/stromal cells (MSCs). *Proc Natl Acad Sci USA* (2016) 113:E6447–56. doi: 10.1073/pnas.1612290113
153. Lu X, Mu E, Wei Y, Riethdorf S, Yang Q, Yuan M, et al. VCAM-1 Promotes Osteolytic Expansion of Indolent Bone Micrometastasis of Breast Cancer by Engaging α4β1-Positive Osteoclast Progenitors. *Cancer Cell* (2011) 20:701–14. doi: 10.1016/j.ccr.2011.11.002
154. Han HH, Kim BG, Lee JH, Kang S, Kim JE, Cho NH. Angiopoietin-2 promotes ER+ breast cancer cell survival in bone marrow niche. *Endocr Relat Cancer* (2016) 23:609–23. doi: 10.1530/ERC-16-0086
155. Lan Q, Peyvandi S, Duffey N, Huang YT, Barras D, Held W, et al. Type I interferon/IRF7 axis instigates chemotherapy-induced immunological dormancy in breast cancer. *Oncogene* (2019) 38:2814–29. doi: 10.1038/s41388-018-0624-2
156. Almog N, Ma L, Raychowdhury R, Schwager C, Erber R, Short S, et al. Transcriptional switch of dormant tumors to fast-growing angiogenic phenotype. *Cancer Res* (2009) 69:836–44. doi: 10.1158/0008-5472.CAN-08-2590
157. Li Y, Hu H, Wang Y, Fan Y, Yang Y, Guo B, et al. CUL4B contributes to cancer stemness by repressing tumor suppressor miR34a in colorectal cancer. *Oncogenesis* (2020) 9. doi: 10.1038/s41389-020-0206-3
158. Tiram G, Ferber S, Ofek P, Eldar-Boock A, Ben-Shushan D, Yeini E, et al. Reverting the molecular fingerprint of tumor dormancy as a therapeutic strategy for glioblastoma. *FASEB J* (2018) 32:5835–50. doi: 10.1096/fj.201701568R
159. Magnus N, Garnier D, Meehan B, McGraw S, Lee TH, Caron M, et al. Tissue factor expression provokes escape from tumor dormancy and leads to genomic alterations. *Proc Natl Acad Sci USA* (2014) 111:3544–9. doi: 10.1073/pnas.1314118111
160. Almog N, Briggs C, Beheshti A, Ma L, Wilkie KP, Rietman E, et al. Transcriptional changes induced by the tumor dormancy-associated microRNA-190. *Transcription* (2013) 4:177–91. doi: 10.4161/trns.25558
161. Jiang J, Zheng M, Zhang M, Yang X, Li L, Wang SS, et al. PRRX1 Regulates Cellular Phenotype Plasticity and Dormancy of Head and Neck Squamous Cell Carcinoma Through miR-642b-3p. *Neoplasia (United States)* (2019) 21:216–29. doi: 10.1016/j.neo.2018.12.001
162. Aguirre Ghiso JA, Kovalski K, Ossowski L. Tumor dormancy induced by downregulation of urokinase receptor in human carcinoma involves integrin and MAPK signaling. *J Cell Biol* (1999) 147:89–103. doi: 10.1083/jcb.147.1.89
163. Schewe DM, Aguirre-Ghiso JA. ATF6α-Rheb-mTOR signaling promotes survival of dormant tumor cells in vivo. *Proc Natl Acad Sci USA* (2008) 105:10519–24. doi: 10.1073/pnas.0800939105
164. Yang LY, He CY, Chen XH, Su LP, Liu BY, Zhang H. Aurora kinase A revives dormant laryngeal squamous cell carcinoma cells via FAK/PI3K/Akt pathway activation. *Oncotarget* (2016) 7:48346–59. doi: 10.18632/oncotarget.10233
165. Shachaf CM, Kopelman AM, Arvanitis C, Karlsson Å, Beer S, Mandl S, et al. MYC inactivation uncovers pluripotent differentiation and tumour dormancy in hepatocellular cancer. *Nature* (2004) 431:1112–7. doi: 10.1038/nature03043
166. Kurppa KJ, Liu Y, To C, Zhang T, Fan M, Vajdi A, et al. Treatment-Induced Tumor Dormancy through YAP-Mediated Transcriptional Reprogramming of the Apoptotic Pathway. *Cancer Cell* (2020) 37:104–22.e12. doi: 10.1016/j.ccell.2019.12.006
167. Liang X, Gu J, Li TJ, Zhao L, Fu X, Zhang W, et al. PAX5 haploinsufficiency induce cancer cell dormancy in Raji cells. *Exp Cell Res* (2018) 367:30–6. doi: 10.1016/j.yexcr.2018.02.010
168. Nash KT, Phadke PA, Navenot JM, Hurst DR, Accavitti-Loper MA, Sztul E, et al. Requirement of KISS1 secretion for multiple organ metastasis suppression and maintenance of tumor dormancy. *J Natl Cancer Inst* (2007) 99:309–21. doi: 10.1093/jnci/djk053
169. Jia Q, Yang F, Huang W, Zhang Y, Bao B, Li K, et al. Low levels of sox2 are required for melanoma tumor-repopulating cell dormancy. *Theranostics* (2019) 9:424–35. doi: 10.7150/thno.29698
170. Liu Y, Liang X, Yin X, Lv J, Tang K, Ma J, et al. Blockade of IDO-kynurenine-AhR metabolic circuitry abrogates IFN-γ-induced immunologic dormancy of tumor-repopulating cells. *Nat Commun* (2017) 8. doi: 10.1038/ncomms15207
171. Ambs S, Dennis S, Fairman J, Wright M, Papkoff J. Inhibition of tumor growth correlates with the expression level of a human angiostatin transgene in transfected B16f10 melanoma cells. *Cancer Res* (1999) 59:5773–7.
172. Malafa MP, Fokum FD, Smith LK, Louis A. Inhibition of angiogenesis and promotion of melanoma dormancy by vitamin E succinate. *Ann Surg Oncol* (2002) 9:1023–32. doi: 10.1245/ASO.2002.03.580
173. Touil Y, Segard P, Ostyn P, Begard S, Aspord C, El MacHhour R, et al. Melanoma dormancy in a mouse model is linked to GILZ/FOXO3A-dependent quiescence of disseminated stem-like cells. *Sci Rep* (2016) 6. doi: 10.1038/srep30405
174. Panigrahy D, Edin ML, Lee CR, Huang S, Bielenberg DR, Butterfield CE, et al. Epoxyeicosanoids stimulate multiorgan metastasis and tumor dormancy escape in mice. *J Clin Invest* (2012) 122:178–91. doi: 10.1172/JCI58128
175. Dossi R, Frapolli R, Di Giandomenico S, Paracchini L, Bozzi F, Brich S, et al. Antiangiogenic activity of trabectedin in myxoid liposarcoma: Involvement of host TIMP-1 and TIMP-2 and tumor thrombospondin-1. *Int J Cancer* (2015) 136:721–9. doi: 10.1002/ijc.29023
176. Chen H, Ko JMY, Wong VCL, Hyytiainen M, Keski-Oja J, Chua D, et al. LTBP-2 confers pleiotropic suppression and promotes dormancy in a growth factor permissive microenvironment in nasopharyngeal carcinoma. *Cancer Lett* (2012) 325:89–98. doi: 10.1016/j.canlet.2012.06.005
177. Tiram G, Segal E, Krivitsky A, Shreberk-Hassidim R, Ferber S, Ofek P, et al. Identification of Dormancy-Associated MicroRNAs for the Design of Osteosarcoma-Targeted Dendritic Polyglycerol Nanopolyplexes. *ACS Nano* (2016) 10:2028–45. doi: 10.1021/acsnano.5b06189
178. Lyu T, Jia N, Wang J, Yan X, Yu Y, Lu Z, et al. Expression and epigenetic regulation of angiogenesis-related factors during dormancy and recurrent growth of ovarian carcinoma. *Epigenetics* (2013) 8:1330–46. doi: 10.4161/epi.26675
179. Mao W, Peters HL, Sutton MN, Orozco AF, Pang L, Yang H, et al. The role of vascular endothelial growth factor, interleukin 8, and insulinlike growth factor in sustaining autophagic DIRAS3-induced dormant ovarian cancer xenografts. *Cancer* (2019) 125:1267–80. doi: 10.1002/cncr.31935
180. Lu Z, Luo RZ, Lu Y, Zhang X, Yu Q, Khare S, et al. The tumor suppressor gene ARHI regulates autophagy and tumor dormancy in human ovarian cancer cells. *J Clin Invest* (2008) 118:3917–29. doi: 10.1172/JCI35512
181. Luo XL, Deng CC, Su XD, Wang F, Chen Z, Wu XP, et al. Loss of MED12 induces tumor dormancy in human epithelial ovarian cancer via

downregulation of EGFR. *Cancer Res* (2018) 78:3532–43. doi: 10.1158/0008-5472.CAN-18-0134
182. Lee HH, Bellat V, Law B. Chemotherapy induces adaptive drug resistance and metastatic potentials via phenotypic CXCR4-expressing cell state transition in ovarian cancer. *PloS One* (2017) 12. doi: 10.1371/journal.pone.0171044
183. Rajbhandari N, Lin Wc, Wehde BL, Triplett AA, Wagner KU. Autocrine IGF1 Signaling Mediates Pancreatic Tumor Cell Dormancy in the Absence of Oncogenic Drivers. *Cell Rep* (2017) 18:2243–55. doi: 10.1016/j.celrep.2017.02.013
184. Lenk L, Pein M, Will O, Gomez B, Viol F, Hauser C, et al. The hepatic microenvironment essentially determines tumor cell dormancy and metastatic outgrowth of pancreatic ductal adenocarcinoma. *Oncoimmunology* (2018) 7:1–14. doi: 10.1080/2162402X.2017.1368603
185. Tang Y, Wang MT, Chen Y, Yang D, Che M, Honn KV, et al. Downregulation of vascular endothelial growth factor and induction of tumor dormancy by 15-lipoxygenase-2 in prostate cancer. *Int J Cancer* (2009) 124:1545–51. doi: 10.1002/ijc.24118
186. Cackowski F, Eber MR, Rhee J, Decker A, Yumoto K, Berry JE, et al. Mer Tyrosine Kinase Regulates Disseminated Prostate Cancer Cellular Dormancy Graphical Abstract HHS Public Access. *J Cell Biochem* (2017) 118:891–902. doi: 10.1002/jcb.25768
187. Komatsu M, Kurokawa H, Waguri S, Taguchi K, Kobayashi A, Ichimura Y, et al. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat Cell Biol* (2010) 12:213–23. doi: 10.1038/ncb2021
188. Axelrod HD, Valkenburg KC, Amend SR, Hicks JL, Parsana P, Torga G, et al. AXL is a putative tumor suppressor and dormancy regulator in prostate cancer. *Mol Cancer Res* (2019) 17:356–69. doi: 10.1158/1541-7786.MCR-18-0718
189. Kim JK, Jung Y, Wang J, Joseph J, Mishra A, Hill EE, et al. TBK1 regulates prostate cancer dormancy through mTOR inhibition. *Neoplasia (United States)* (2013) 15:1064–74. doi: 10.1593/neo.13402
190. O'Reilly MS, Holmgren L, Chen C, Folkman J. Angiostatin induces and sustains dormancy of human primary tumors in mice. *Nat Med* (1996) 2:689–92. doi: 10.1038/nm0696-689
191. Phan TG, Croucher PI. The dormant cancer cell life cycle. *Nat Rev Cancer* (2020) 20:398–411. doi: 10.1038/s41568-020-0263-0
192. Aguirre-Ghiso JA, Sosa MS. Emerging Topics on Disseminated Cancer Cell Dormancy and the Paradigm of Metastasis. *Annu Rev Cancer Biol* (2018) 2:377–93. doi: 10.1146/annurev-cancerbio-030617-050446
193. Mortensen M, Soilleux EJ, Djordjevic G, Tripp R, Lutteropp M, Sadighi-Akha E, et al. The autophagy protein Atg7 is essential for hematopoietic stem cell maintenance. *J Exp Med* (2011) 208:455–67. doi: 10.1084/jem.20101145
194. Lazova R, Camp RL, Klump V, Siddiqui SF, Amaravadi RK, Pawelek JM. Punctate LC3B expression is a common feature of solid tumors and associated with proliferation, metastasis, and poor outcome. *Clin Cancer Res* (2012) 18:370–9. doi: 10.1158/1078-0432.CCR-11-1282
195. Yang A, Rajeshkumar NV, Wang X, Yabuuchi S, Alexander BM, Chu GC, et al. Autophagy is critical for pancreatic tumor growth and progression in tumors with p53 alterations. *Cancer Discovery* (2014) 10:1683–4. doi: 10.1158/2159-8290.CD-14-0362
196. Cianfanelli V, Fuoco C, Lorente M, Salazar M, Quondamatteo F, Gherardini PF, et al. AMBRA1 links autophagy to cell proliferation and tumorigenesis by promoting c-Myc dephosphorylation and degradation. *Nat Cell Biol* (2015) 17:20–30. doi: 10.1038/ncb3072
197. Mowers EE, Sharifi MN, Macleod KF. Autophagy in cancer metastasis. *Oncogene* (2017) 36:1619–30. doi: 10.1038/onc.2016.333
198. Gupta A, Roy S, Lazar AJF, Wang WL, McAuliffe JC, Reynoso D, et al. Autophagy inhibition and antimalarials promote cell death in gastrointestinal stromal tumor (GIST). *Proc Natl Acad Sci USA* (2010) 107:14333–8. doi: 10.1073/pnas.1000248107
199. Vera-Ramirez L, Vodnala SK, Nini R, Hunter KW, Green JE. Autophagy promotes the survival of dormant breast cancer cells and metastatic tumour recurrence. *Nat Commun* (2018) 9. doi: 10.1038/s41467-018-04070-6
200. Yu Z, Zhou R, Zhao Y, Pan Y, Liang H, Zhang JS, et al. Blockage of SLC31A1-dependent copper absorption increases pancreatic cancer cell autophagy to resist cell death. *Cell Prolif* (2019) 52. doi: 10.1111/cpr.12568
201. Lu Z, Baquero MT, Yang H, Yang M, Reger AS, Kim C, et al. DIRAS3 regulates the autophagosome initiation complex in dormant ovarian cancer cells. *Autophagy* (2014) 10:1071–92. doi: 10.4161/auto.28577
202. Washington MN, Suh G, Orozco AF, Sutton MN, Yang H, Wang Y, et al. ARHI (DIRAS3)-mediated autophagy-associated cell death enhances chemosensitivity to cisplatin in ovarian cancer cell lines and xenografts. *Cell Death Dis* (2015) 6. doi: 10.1038/cddis.2015.208
203. Bao JJ, Le XF, Wang RY, Yuan J, Wang L, Atkinson EN, et al. Reexpression of the tumor suppressor gene ARHI induces apoptosis in ovarian and breast cancer cells through a caspase-independent calpain-dependent pathway. *Cancer Res* (2002) 62:7264–72.
204. Correa RJM, Valdes YR, Peart TM, Fazio EN, Bertrand M, McGee J, et al. Combination of AKT inhibition with autophagy blockade effectively reduces ascites-derived ovarian cancer cell viability. *Carcinogenesis* (2014) 35:1951–61. doi: 10.1093/carcin/bgu049
205. Peart T, Valdes YR, Correa RJM, Fazio E, Bertrand M, McGee J, et al. Intact LKB1 activity is required for survival of dormant ovarian cancer spheroids. *Oncotarget* (2015) 6:22424–38. doi: 10.18632/oncotarget.4211
206. Carcereri de Prati A, Butturini E, Rigo A, Oppici E, Rossin M, Boriero D, et al. Metastatic Breast Cancer Cells Enter Into Dormant State and Express Cancer Stem Cells Phenotype Under Chronic Hypoxia.s. *J Cell Biochem* (2017) 118:3237–48. doi: 10.1002/jcb.25972
207. Aqbi HF, Tyutyunyk-Massey L, Keim RC, Butler SE, Thekkudan T, Joshi S, et al. Autophagy-deficient breast cancer shows early tumor recurrence and escape from dormancy. *Oncotarget* (2018) 9:22128–37. doi: 10.18632/oncotarget.25197
208. Shinde A, Hardy SD, Kim D, Akhand SS, Jolly MK, Wang WH, et al. Spleen tyrosine kinase–mediated autophagy is required for epithelial–mesenchymal plasticity and metastasis in breast cancer. *Cancer Res* (2019) 79:1831–43. doi: 10.1158/0008-5472.CAN-18-2636
209. Wang L, Hoque A, Luo RZ, Yuan J, Lu Z, Nishimoto A, et al. Loss of the expression of the tumor suppressor gene ARHI is associated with progression of breast cancer. *Clin Cancer Res* (2003) 9:3660–6.
210. Lu Z, Luo RZ, Peng H, Rosen DG, Atkinson EN, Warneke C, et al. Transcriptional and posttranscriptional down-regulation of the imprinted tumor suppressor gene ARHI (DRAS3) in ovarian cancer. *Clin Cancer Res* (2006) 12:2404–13. doi: 10.1158/1078-0432.CCR-05-1036
211. Zou CF, Jia L, Jin H, Yao M, Zhao N, Huan J, et al. Re-expression of ARHI (DIRAS3) induces autophagy in breast cancer cells and enhances the inhibitory effect of paclitaxel. *BMC Cancer* (2011) 11. doi: 10.1186/1471-2407-11-22
212. Chaterjee M, van Golen KL. Breast Cancer Stem Cells Survive Periods of Farnesyl-Transferase Inhibitor-Induced Dormancy by Undergoing Autophagy. *Bone Marrow Res* (2011) 2011:1–7. doi: 10.1155/2011/362938
213. Ornelas A, McCullough CR, Lu Z, Zacharias NM, Kelderhouse LE, Gray J, et al. Induction of autophagy by ARHI (DIRAS3) alters fundamental metabolic pathways in ovarian cancer models. *BMC Cancer* (2016) 16. doi: 10.1186/s12885-016-2850-8
214. Kamada Y, Funakoshi T, Shintani T, Nagano K, Ohsumi M, Ohsumi Y. Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J Cell Biol* (2000) 150:1507–13. doi: 10.1083/jcb.150.6.1507
215. Ravikumar B, Vacher C, Berger Z, Davies JE, Luo S, Oroz LG, et al. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat Genet* (2004) 36:585–95. doi: 10.1038/ng1362
216. Takeuchi H, Kondo Y, Fujiwara K, Kanzawa T, Aoki H, Mills GB, et al. Synergistic augmentation of rapamycin-induced autophagy in malignant glioma cells by phosphatidylinositol 3-kinase/protein kinase B inhibitors. *Cancer Res* (2005) 65:3336–46. doi: 10.1158/0008-5472.CAN-04-3640
217. Tanemura M, Ohmura Y, Deguchi T, MacHida T, Tsukamoto R, Wada H, et al. Rapamycin causes upregulation of autophagy and impairs islets function both in vitro and in vivo. *Am J Transplant* (2012) 12:102–14. doi: 10.1111/j.1600-6143.2011.03771.x
218. Corradetti MN, Inoki K, Bardeesy N, DePinho RA, Guan KL. Regulation of the TSC pathway by LKB1: Evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. *Genes Dev* (2004) 18:1533–8. doi: 10.1101/gad.1199104
219. Mungai PT, Waypa GB, Jairaman A, Prakriya M, Dokic D, Ball MK, et al. Hypoxia Triggers AMPK Activation through Reactive Oxygen Species-Mediated Activation of Calcium Release-Activated Calcium Channels. *Mol Cell Biol* (2011) 31:3531–45. doi: 10.1128/mcb.05124-11

220. Meijer AJ, Codogno P. Regulation and role of autophagy in mammalian cells. *Int J Biochem Cell Biol* (2004) 36:2445–62. doi: 10.1016/j.biocel.2004.02.002
221. Inoki K, Li Y, Xu T, Guan KL. Rheb GTpase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev* (2003) 17:1829–34. doi: 10.1101/gad.1110003
222. Lu Z, Yang H, Sutton MN, Yang M, Clarke CH, Liao WSL, et al. ARHI (DIRAS3) induces autophagy in ovarian cancer cells by downregulating the epidermal growth factor receptor, inhibiting PI3K and Ras/MAP signaling and activating the FOXo3a-mediated induction of Rab7. *Cell Death Differ* (2014) 21:1275–89. doi: 10.1038/cdd.2014.48
223. Palmieri M, Impey S, Kang H, di Ronza A, Pelz C, Sardiello M, et al. Characterization of the CLEAR network reveals an integrated control of cellular clearance pathways. *Hum Mol Genet* (2011) 20:3852–66. doi: 10.1093/hmg/ddr306
224. Settembre C, Di Malta C, Polito VA, Arencibia MG, Vetrini F, Erdin S, et al. TFEB links autophagy to lysosomal biogenesis. *Science (80-)* (2011) 332:1429–33. doi: 10.1126/science.1204592
225. Sutton MN, Huang GY, Zhou J, Mao W, Langley R, Lu Z, et al. Amino acid deprivation-induced autophagy requires upregulation of DIRAS3 through reduction of E2F1 and E2F4 transcriptional repression. *Cancers (Basel)* (2019) 11. doi: 10.3390/cancers11050603
226. Blancato J, Graves A, Rashidi B, Moroni M, Tchobe L, Ozdemirli M, et al. SYK allelic loss and the role of Syk-regulated genes in breast cancer survival. *PloS One* (2014) 9. doi: 10.1371/journal.pone.0087610
227. Krisenko MO, Higgins RL, Ghosh S, Zhou Q, Trybula JS, Wang WH, et al. Syk is recruited to stress granules and promotes their clearance through autophagy. *J Biol Chem* (2015) 290:27803–15. doi: 10.1074/jbc.M115.642900
228. Du H, Yang W, Chen L, Shi M, Seewoo V, Wang J, et al. Role of autophagy in resistance to oxaliplatin in hepatocellular carcinoma cells. *Oncol Rep* (2012) 27:143–50. doi: 10.3892/or.2011.1464
229. Shimizu S, Takehara T, Hikita H, Kodama T, Tsunematsu H, Miyagi T, et al. Inhibition of autophagy potentiates the antitumor effect of the multikinase inhibitor sorafenib in hepatocellular carcinoma. *Int J Cancer* (2012) 131:548–57. doi: 10.1002/ijc.26374
230. Qu X, Sheng J, Shen L, Su J, Xu Y, Xie Q, et al. Autophagy inhibitor chloroquine increases sensitivity to cisplatin in QBC939 cholangiocarcinoma cells by mitochondrial ROS. *PloS One* (2017) 12. doi: 10.1371/journal.pone.0173712
231. Tong M, Che N, Zhou L, Luk ST, Kau PW, Chai S, et al. Efficacy of annexin A3 blockade in sensitizing hepatocellular carcinoma to sorafenib and regorafenib. *J Hepatol* (2018) 69:826–39. doi: 10.1016/j.jhep.2018.05.034
232. Blessing AM, Santiago-O'Farrill JM, Mao W, Pang L, Ning J, Pak D, et al. Elimination of dormant, autophagic ovarian cancer cells and xenografts through enhanced sensitivity to anaplastic lymphoma kinase inhibition. *Cancer* (2020). doi: 10.1002/cncr.32985
233. Nishikawa S, Ishii H, Haraguchi N, Kano Y, Fukusumi T, Ohta K, et al. Genotoxic therapy stimulates error-prone DNA repair in dormant hepatocellular cancer stem cells. *Exp Ther Med* (2012) 3:959–62. doi: 10.3892/etm.2012.522
234. Evans EB, Lin SY. New insights into tumor dormancy: Targeting DNA repair pathways. *World J Clin Oncol* (2015) 6:80–8. doi: 10.5306/wjco.v6.i5.80
235. Skvortsova I, Debbage P, Kumar V, Skvortsov S. Radiation resistance: Cancer stem cells (CSCs) and their enigmatic pro-survival signaling. *Semin Cancer Biol* (2015) 35:39–44. doi: 10.1016/j.semcancer.2015.09.009
236. Beerman I, Seita J, Inlay MA, Weissman IL, Rossi DJ. Quiescent hematopoietic stem cells accumulate DNA damage during aging that is repaired upon entry into cell cycle. *Cell Stem Cell* (2014) 15:37–50. doi: 10.1016/j.stem.2014.04.016
237. Yalcin A, Clem BF, Simmons A, Lane A, Nelson K, Clem AL, et al. Nuclear targeting of 6-phosphofructo-2-kinase (PFKFB3) increases proliferation via cyclin-dependent kinases. *J Biol Chem* (2009) 284:24223–32. doi: 10.1074/jbc.M109.016816
238. Settembre C, Fraldi A, Medina DL, Ballabio A. Signals from the lysosome: A control centre for cellular clearance and energy metabolism. *Nat Rev Mol Cell Biol* (2013) 14:283–96. doi: 10.1038/nrm3565

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Akkoc, Peker, Akcay and Gozuacik. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Oncology

MINI REVIEW
published: 31 March 2021
doi: 10.3389/fonc.2021.641269

Autophagy in Human T-Cell Leukemia Virus Type 1 (HTLV-1) Induced Leukemia

Nicolás Ducasa[1], Daniel Grasso[2,3], Paula Benencio[1], Daniela L. Papademetrio[3,4], Mirna Biglione[1], Fatah Kashanchi[5], Carolina Berini[1†] and Maria Noé Garcia[3,4†]*

[1] Instituto de Investigaciones Biomédicas en Retrovirus y SIDA (INBIRS), CONICET- Universidad de Buenos Aires, Buenos Aires, Argentina, [2] Cátedra de Fisiopatología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina, [3] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Instituto de Estudios de la Inmunidad Humoral (IDEHU), Buenos Aires, Argentina, [4] Cátedra de Inmunología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina, [5] Laboratory of Molecular Virology, School of Systems Biology, George Mason University, Manassas, VA, United States

OPEN ACCESS

Edited by:
César López-Camarillo, Universidad Autónoma de la Ciudad de México, Mexico

Reviewed by:
Kazuhiro Morishita, University of Miyazaki, Japan
Eloy Andres Perez Yepez, National Cancer Institute, Mexico

***Correspondence:**
Maria Noé Garcia
mn.garcia@docente.ffyb.uba.ar

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: 14 December 2020
Accepted: 10 March 2021
Published: 31 March 2021

Citation:
Ducasa N, Grasso D, Benencio P, Papademetrio DL, Biglione M, Kashanchi F, Berini C and Garcia MN (2021) Autophagy in Human T-Cell Leukemia Virus Type 1 (HTLV-1) Induced Leukemia. Front. Oncol. 11:641269. doi: 10.3389/fonc.2021.641269

Viruses play an important role in the development of certain human cancers. They are estimated to contribute 16% to all human cancers. Human T-cell leukemia virus type 1 (HTLV-1) was the first human retrovirus to be discovered and is the etiological agent of adult T-cell leukemia/lymphoma (ATLL), an aggressive T-cell malignancy with poor prognosis. HTLV-1 viral proteins interact with mechanisms and proteins present in host cells for their own benefit, evading the immune system and promoting the establishment of disease. Several viruses manipulate the autophagy pathway to achieve their infective goals, and HTLV-1 is not the exception. HTLV-1 Tax viral protein engages NF-κB and autophagy pathways prone favoring viral replication and T cell transformation. In this review we focus on describing the relationship of HTLV-1 with the autophagy machinery and its implication in the development of ATLL.

Keywords: HTLV-1, autophagy, T-cell leukemia, NF-κB, tax

INTRODUCTION

Human T cell leukemia virus type 1 (HTLV-1), was the first human retrovirus discovered (1). It is the etiological agent of an aggressive T cell malignancy known as adult T cell leukemia/lymphoma (ATLL) and a neurologic disease named HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), other inflammatory syndromes, opportunistic infections, and lung diseases (2). HTLV-1 is transmitted through sexual contact, from mother to child (mainly by prolonged breastfeeding) and parenterally (3, 4). In 2014, HTLV-1 was included as group 1 human carcinogens by the International Agency for Research on Cancer (IARC) (5). The vast majority of HTLV-1 infected individuals are asymptomatic and around 3-5% of them will develop ATLL, that usually occurs after a long latency period. It is clinically classified as smoldering, chronic, lymphoma and acute (6). The smoldering and chronic without unfavorable prognostic factors are categorized as indolent ATLL and generally progress slowly. On the contrary, acute, lymphoma and chronic with unfavorable prognostic factors are aggressive forms and patients have a survival of months (7, 8). The HTLV-1 genome shares the structural features of other retroviruses, but it also has a pX region which

encodes regulatory proteins, such as Tax and bZIP factor (HBZ) (9). HBZ and Tax have opposing functions in most transcription pathways, but both proteins play a critical role in HTLV-1 infection as well as in growth and survival of leukemia cells (10).

Autophagy (also known as macroautophagy) is a degradative process for cellular components including macromolecules such as proteins, RNA and even whole organelles (11). Under stress conditions such as cell starvation, inhibition of mTORC1 (mammalian Target of Rapamycin Complex 1) leads the activation of ULK1 (unc-51 Like Autophagy Activating Kinase 1) complex which in turn triggers the autophagosome biogenesis (12). ULK1 activates a complex which includes BECN1 among its members and a PI3KC3 (phosphatidylinositol 3-kinase Class 3). The PI3P (phosphatidylinositol 3-phosphate) generated by this last complex is required for recruitment of further autophagic proteins and the autophagosome formation. Autophagosomes are double membrane vesicles decorated by LC3 protein and, once it is loaded with the cargo, this particular vesicle is carried to fuse with lysosomes where the cargo is eventually degraded (12–15).

Alternatively, autophagosomes can fuse with components of the endosomal system, late endosomes or multivesicular bodies (MVBs) (16). These hybrid compartments, named as amphisomes, have the options of degrading its intravesicular material by fusion with a lysosome or fusing with the plasma membrane (17, 18). In the case they fuse with the plasma membrane, their contents are released outside the cell including extracellular vesicles (EVs) (19, 20). These small vesicles have recently gained special relevance since they are important intercellular messengers capable of carrying several molecules, proteins, nucleic acid, and even viral components, and yield an effector response in the target cell (21–24). Importantly, exciting new data is supporting the idea of a superlative crosstalk between autophagy and EVs machineries (25–30). Moreover, autophagy can take an antiviral or pro-viral role. It could be expected to degrade intracellular pathogens, but certain viruses have evolved to use the autophagic machinery for their own benefit, increasing viral replication and viral spread (31).

ADULT T-CELL LEUKEMIA/LYMPHOMA

The adult T-cell leukemia/lymphoma or ATLL is a malignant and aggressive neoplasm as a consequence of HTLV-1 infection. In endemic regions of Japan, ATLL affects about 8.7 persons per 10,000 HTLV-1 infected citizens and, having in mind an annual incidence of 20 million around the world, it is expected a significant number of persons suffering this pathology (32). In Latin America, 1% of HTLV-1 infected individuals are asymptomatic positive, but in some endemic areas it might reach 10% (32). It is interesting to know that HTLV-1 infects T cells, B cells, fibroblasts, dendritic cells, and macrophages, though hitherto data show it is only capable of transforming regulatory T cells which are positive for CD4/CD25 (33). The carcinogenesis induced by HTLV-1 infection possesses a biphasic behavior, with an initiation and a maintenance phases. Epidemiology demonstrates that ATLL onset is observed about the fifth decade in individuals that were infected during the firsts years of life (34, 35). This suggests an extensive latency period coincident with the conception that oncogenesis is initiated in a first phase of viral infection and then a second phase where oncogenic properties of transformed cells are maintained (34, 35).

HTLV-1 seems to rely on two main proteins for cellular transformation, HBZ and Tax. Data suggests that HBZ is important for viral replication, cellular proliferation and evasion from the immune system (36, 37). Then, the major key role of HBZ in oncogenesis is maintaining the oncogenic phenotype by attenuation of host immune response against leukemic cells and fostering a microenvironment appropriate for HTLV-1 infected cells (38). On the other hand, Tax is the main actor for the T cells transformation process engaging several cellular pathways (39). This means that Tax appears early during infection and during the long period of latency time, and it is crucial to initiate cellular transformation. Once cells are transformed, HBZ enters in the second phase for maintaining the transformed phenotype. This is also supported by the fact that Tax gradually disappears during that time, to the point of being almost undetectable, contrary to HBZ whose presence is prominent and ubiquitous in advanced stages of ATLL (40, 41).

The central role of Tax in ATLL is highlighted with the observation that impairment of functionality of *tax* gene impedes T cells transformation (42). Even more, Tax overexpression provokes a leukemia phenotype in transgenic mouse models (43, 44). This data suggests that Tax is indeed enough for T cells immortalization. Moreover, the capacity of Tax to induce cell survival, proliferation and bypass tumor suppressor processes such as senescence and apoptosis has been vastly demonstrated (45–50). The powerful property of Tax lies on its ability to activate a myriad of signaling pathways including PI3K/AKT, p53 inhibition, induction of ROS (reactive oxygen species) production and even genome instability by direct DNA damage and impairment of proteins related to DNA repair (51, 52). Although, those characteristics are important in Tax-mediated transformation is worthy to mention specially the NF-κB pathway. Tax has the ability to activate both canonical and noncanonical NF-κB pathways which in turn set up a broad cell survival program (53–55). Tax-mediated initial activation of NF-κB pathways is in such a way that it persists even after Tax expression has disappeared (52, 56).

HTLV-1 TAX RELATIONSHIP WITH THE NF-ΚB PATHWAY

The NF-κB family of transcription factors is composed of five members: RelA (p65), c-Rel, RelB, NF-κB1 (p50) and NF-κB2 (p52), which can form hetero or homodimeric combinations (57). There are two major pathways for NF-κB activation: the canonical and non-canonical NF-κB signaling pathways. Canonical NF-κB signaling is induced upon stimulation by pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, pathogen-associated molecular patterns (PAMPs) from viruses and bacteria, agonists for the B or T cell antigen receptors (BCR or TCR), and chemicals or radiation (58). On the other hand, non-canonical NF-κB signaling pathway is restricted to a subset of TNF family members such as B cell activating factor (BAFF), lymphotoxinβ-(LTβ) and CD40L (59). These two pathways of NF-κB activation differ, not only in the involved receptors, but also, in the implicated molecules and the

generated response. The induction of the canonical NF-κB signaling involves a variety of different adaptor molecules to engage the IKK complex which in turn triggers the signaling pathway (60). IKK complex consists of the regulatory subunit IKKγ/NEMO, and IKKα and IKKβ, the catalytic ones (61). Once activated, IKK phosphorylates IκBs subunits (IκBα, IκBβ and IκBε) inducing the IκBs ubiquitination and proteasomal degradation (62). Then, classical NF-κB dimers, like p50/RelA and p50/c-Rel, are released from IκB to enter the nucleus and induce the transcription of target genes (60). This activation of the canonical NF-κB pathway under physiological conditions, induces a rapid but transient transcriptional response (58, 59). On the contrary, non-canonical NF-κB signaling activation relies on NIK, which in resting cells is constantly degraded by an E3 ligase complex consisting of the E3 ligases c-IAP1/2 and the adaptor TRAF3/TRAF2. Activation of BAFFR, LTβR and CD40 provokes inactivation of the TRAF/c-IAP complex and the consequent NIK stabilization. In this situation, NIK phosphorylates IKK, which in turn phosphorylates p100/RelB tagging it for proteasomal processing and the consequent release of p52/RelB, which translocates to the nucleus. Compared to the canonical way, non-canonical NF-κB response is delayed, but its transcriptional response is sustained in time (58, 59). It has been described the existence of negative regulators of the NF-κB pathway that could be involved in the constitutive activation of NF-κB in ATLL such as TNF-α-induced protein 3 (TNFAIP3, A20), Cylindromatosis (CYLD), and NSFL1 cofactor (p47) among others. The implication of any of these negative regulators of the NF-κB pathway could be of extreme importance in the persistence of its activation (63–65).

In HTLV-1 infection, Tax persistently activates both canonical and non-canonical NF-κB pathways which are required for cell survival and T lymphocyte transformation (66, 67). Indeed, a persistent NF-κB activity is observed in HTLV-1 transformed cell lines (54). By intervention at different levels, Tax ensures the NF-κB pathway activation without external signals. HTLV-1 Tax interacts with TAK1-binding protein 2 (TAB-2) activating MEKK1 and TAK1 which in turn activate the IKK complex (68, 69) (**Figure 1**).

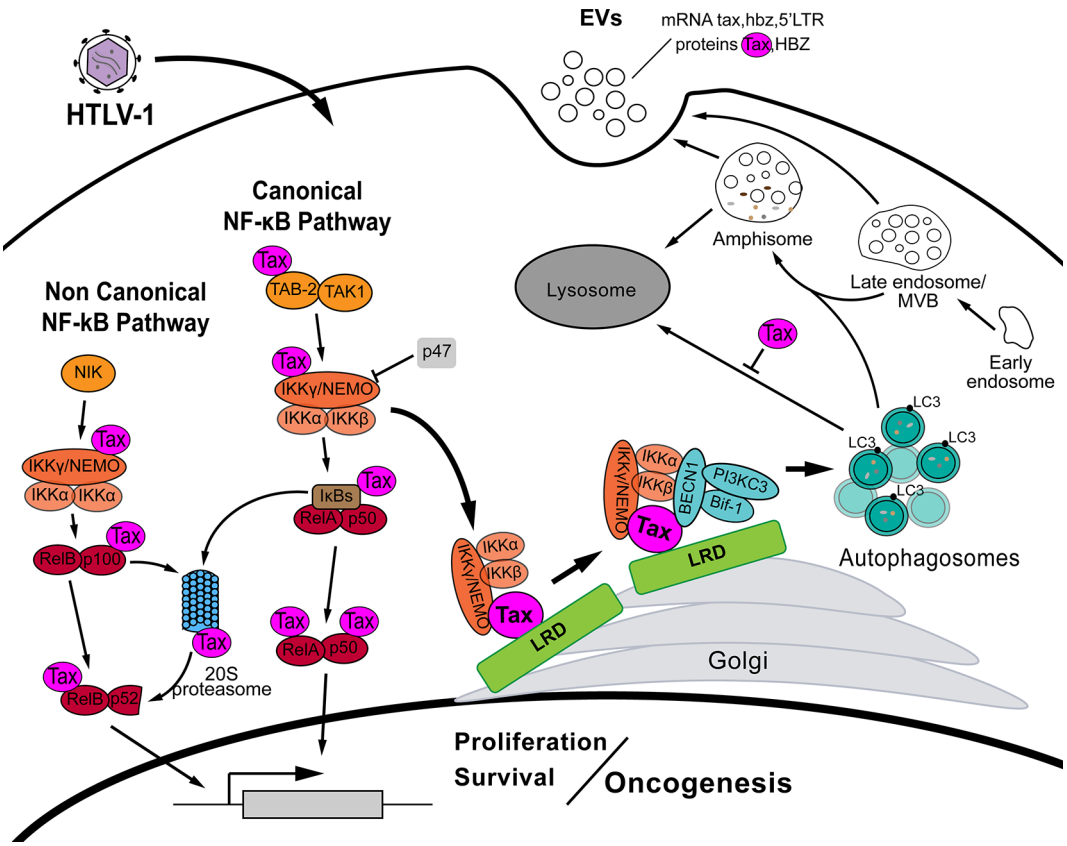


FIGURE 1 | In HTLV-1 infection, the viral protein Tax interferes at several steps of both canonical and noncanonical NF-κB pathway in order to activate it, inducing cell survival and proliferation, and eventually resulting in oncogenesis. By interaction with IKKγ/NEMO, Tax recruits and activates IKK complex (IKKγ/NEMO, IKKα, IKKβ) in lipid raft domains (LRD) on the Golgi. After IKK activation, Tax recruits the autophagy proteins BECN1, Bif-1 and the PI3KC3 complex through its direct interaction with BECN1, which in turn binds also with IKKα, IKKβ. Then, Tax deregulates the autophagy pathway fostering autophagosomes biogenesis but, at the same time, blocking the autophagosome-lysosome fusion. Autophagosomes accumulation enhances HTLV-1 replication. Moreover, recent data suggest a crosstalk between autophagic and extracellular vesicles (EVs) biogenesis pathways. EVs from HTLV-1 infected cells bearing the viral proteins Tax and HBZ among some host proteins, and transcriptional mRNA of Tax, HBZ and 5'LTR has been reported.

The direct association of Tax with IKKγ/NEMO in the lipid raft domains (LRD) localized on Golgi is key for Tax goals (70–73). This Tax-IKKγ/NEMO interaction recruits the whole IKK complex and this action is indispensable for its activation (**Figure 1**) (74–76). Moreover, the resulting degradation of IκBs and consequent release of NF-κB transcription factor subunits are further enhanced by Tax direct interaction with IκBs and 20S proteasome (54, 77) (**Figure 1**). On the other hand, Tax also interacts with IKKγ/NEMO and p100 to induce the proteasome-mediated processing of this last in order to activate the non-canonical NF-κB pathway (55, 78–82). These data are supported by the fact that IKK is persistently activated in primary ATLL and HTLV-1 transformed cells (54, 77). The deep involvement of Tax with the NF-κB pathway is justified by the fact that the activity of this pathway is indispensable for T cell transformation and the maintenance of the leukemic phenotype (81, 83).

HTLV-1 TAX DEREGULATION OF AUTOPHAGY

The fight between cells and viruses came from a constant competitive evolution. Cells have developed several strategies against viral infections and autophagy is into their toolkit repertory. For instance, xenophagy and virophagy are two types of selective autophagy that are activated in order to clear intracellular pathogens (84, 85). Xenophagy leads cells to microorganisms recognition, including viruses, to target them towards lysosome, through the autophagy machinery for degradation by the lysosomal hydrolases (84). In a similar way, virophagy tags specific viral components to be degraded by the autophagy flux (86). Nevertheless, viruses also have evolved to evade those strategies and indeed use autophagy machinery for their own benefit (87). In dendritic cells, one strategy of human immunodeficiency virus (HIV-1) is enhancing mTORC1 activation which in turn inhibits the autophagy pathway (88). Another way, used by herpes simplex virus type 1 (HSV-1), is to produce a specific viral protein that suppresses autophagy by its binding to BECN1, which is essential for autophagosome biogenesis initiation (89). Furthermore, most RNA viruses such as hepatitis C virus (HCV) induce autophagy flux to use the double membrane of autophagosome vesicles to hide themselves and, in fact proliferate and come out from the host cell (87, 90, 91). All in all, viruses have developed several strategies with the goal of modifying autophagy in each step, avoiding cellular defensive mechanisms and promoting their proliferation.

It is clear that HTLV-1 infection induces cytoplasmic autophagosomes accumulation and indeed this event increases viral particles production, measured by the viral capsid protein p19 (92). The single transfection of Tax in HeLa and Jurkat cells is enough to accumulate cytoplasmic LC3 positive dots (58). Interestingly, Tax co-localizes with cytoplasmic LC3 puncta but its capacity to accumulate autophagosomes is highly increased when cells are transfected with a Tax targeted by myristoylation to LRDs (83). Worth to note, HTLV-1 capacity to increase cytoplasmic autophagosomes relies also on Tax ability to activate the NF-κB pathway (92). For instance, a mutated Tax without the ability to activate IKK complex is also unable to induce autophagy (83, 93). Besides, impairment of any member of the IKK complex, by abolition of the catalytic activity of IKKα or IKKβ, or the knockdown of IKKγ/NEMO, decreases the cytoplasmic LC3 positive autophagosomes (83). As commented above, Tax activates the IKK complex by recruiting IKKγ/NEMO, and the IKK complex, to the LRD located at the Golgi (71, 73). Additionally, in those LRD, Tax recruits BECN1 and Bif-1, and indeed there is an interaction with PI3KC3 (56, 83). All those proteins belong to the autophagy PI3KC3 complex, the first structural complex of the autophagosome biogenesis (94). In Tax-immortalized T cells Tax co-precipitates with BECN1 and PI3KC3 but not with UVRAG which form with BECN1 another complex related to the autophagosome maturation (95, 96). Without BECN1, Tax is unable to co-precipitated PI3KC3 suggesting that the interaction is through BECN1. Moreover, the sequence of BECN1 that goes from aminoacids 250 to 300 is implicated in the BECN1-Tax interaction (96). Worthy, Tax-mediated recruitment and subsequent activation of IKK complex in the LRDs is a prerequisite to further recruitment of BECN1 and Bif-1 forcing the activation of those autophagy proteins to trigger the autophagosomes biogenesis. This process seems to be exclusive of HTLV-1 infected cells because the co-distribution in LRDs of IKK complex with BECN1 and Bif-1 is only observed in Tax expressing cells (83). Furthermore, the entire IKK complex is key in this Tax-mediated autophagy dysregulation since Tax does not colocalizes in the LRD in absent of IKKγ/NEMO and depletion or impairment of any of the three IKK complex members impedes BECN1 and Bif-1 recruitment to the LRD. The importance of this recruitment is highlighted by the fact that myristoylation of either BECN1 or Bif-1 to target them towards the LRDs induces autophagy (83). It is important to consider the key role that this function of Tax over autophagy seems to have for HTLV-1 infection. Wang and colleagues described how Tax-mediated autophagy provides to infected cells resistance to cell death and, in fact, they suggest to explore autophagy inhibition as a possible treatment against HTLV-1 infection (93).

The relationship between HTLV-1 infection and the autophagy pathway is intricate and the roles of Tax/IKK over the autophagy proteins go in both directions. BECN1 is needed to maintain NF-kB and STAT3 activity in HTLV-1 infected cells (96). STAT3 cooperates with NF-κB in HTLV-1 infected cells. When silencing BECN1, in HTLV-1 transformed cells, a decreased NF-κB and STAT3 activity as well as an impairment in cellular growth is observed (96). Furthermore, PI3KC3 or BECN1 depletion significantly slows the proliferation of HTLV-1 infected T lymphocytes (83). Co-precipitation experiments show that BECN1 interacts directly with the catalytic subunits of IKK complex (i.e. IKKα and IKKβ) through its C-terminal 150 amino acids region. Neither IKKα nor IKKβ alone can co-precipitate BECN1 and the PI3KC3 complex suggesting that both are indispensable for the interaction (60). Altogether, in the LRDs Tax recruits the IKK complex, by its interaction with IKKγ/NEMO, and the autophagy PI3KC3 complex by its interaction

with BECN1. In that context BECN1 interacts with IKKα and IKKβ and it might suggest that LRDs function as a platform where Tax engages the NF-kB and autophagy pathways by the local interaction of Tax, BECN1 and IKK complex (**Figure 1**). Furthermore, as the autophagy is required for the maintenance of NF-κB activity and the LRD recruitment and activation of IKK is needed for Tax-mediated autophagosomes biogenesis, it is logical to speculate about a positive feedback loop between NF-κB and autophagy pathways in HTLV-1 infection (**Figure 1**).

Beyond all above comments, HTLV-1 possesses other goals for autophagy deregulation. The p47 protein was recently found in an attempt to find IKKγ/NEMO interactors. Interestingly, p47 is found highly decreased in HTLV-1 infected cells and in cells from ATLL patients (97). Among its different functions, p47 with its UBA domain is related to degradation of ubiquitinated proteins (98, 99). In CD4+ T lymphocytes, p47 recognizes ubiquitinated IKKγ/NEMO and induces its lysosomal degradation since lysosomal inhibitor but not MG132 (proteasome inhibitor) restore the IKKγ/NEMO levels (97). In this way, p47 negatively regulates IKKγ/NEMO independently of the other two known regulators of the IKK complex, A20 and CYLD (97, 100). Worth to note, shRNA-mediated depletion of p47 significantly potentiates IκBα phosphorylation induced by transfection of Tax in HeLa cells (97). This means that p47 opposes the action of Tax over the NF-κB pathway. In fact, in co-precipitation assay p47 is unable to precipitate IKKγ/NEMO in presence of Tax suggesting that Tax disrupts the p47 binding to IKKγ/NEMO. The regulation of p47 seems to be mediated by its stability since cells from patients with the acute type of ATLL the expression of p47 is similar to uninfected CD4+ T lymphocytes but the amount of p47 protein is significantly lower (65). This degradation of p47 is avoided upon lysosomal inhibition but not with MG132. Moreover, in MEFs $Atg5^{+/+}$ the induction of autophagy by starvation reduces the levels of p47 in stark contrast to $Atg5^{-/-}$ MEFs where the lack of autophagy does not perturb p47 levels upon cell starvation. Finally, similar results were obtained in HTLV-1 infected cells where shRNA-mediated depletion of ATG5 increased the amount of p47, and concomitantly a decrease in IKKγ/NEMO, phosphorylated IκBα and even CADM1 (which is a receptor dependent on NF-κB activity) was detected (65). All these data confirm the degradation of p47 by the autophagy pathway and give an additional reason for Tax-mediated deregulation of the autophagy pathway (**Figure 1**).

The Tax-mediated deregulation of autophagy by Tax/BECN1/IKK in the LRDs is completed with its effects on late steps of autophagosome maturation. Data shows that inhibition of autophagosome-lysosome fusion, by means of bafilomycin A1, improves Tax stability suggesting that Tax could be degraded into the lysosome through the autophagy pathway (92). In consequence, they proved that Tax inhibits the fusion of those degradative vesicles (92). Then, HTLV-1 Tax exerts a deep interference in the autophagy pathway fostering autophagosomes biogenesis but, at the same time, inhibiting the autophagosome-lysosome fusion (**Figure 1**). Additionally, new points of contacts between autophagy and HTLV-1 Tax are still being described such as the case of SQSTM-1/p62. In MEFs and HEK293T cells, but not in Jurkat cells, depletion of SQSTM-1/p62 impair the Tax-mediated NF-κB activity. Indeed, SQSTM-1/p62 directly interacts with Tax in the Tax/IKK complex located in Golgi-associated structures (101). SQSTM-1/p62 is an autophagy receptor with domains for recognition of ubiquitin chains and LC3 to canalize cargoes towards the autophagy-mediated degradation (102, 103). Similar data is obtained with Optineurin, another autophagy selective receptor, but interestingly in both cases Tax interaction with those proteins seems to be related to Tax-mediated NF-κB activation and not with Tax degradation (101, 104). By the side of HBZ, it negatively regulates the autophagy pathway. In cytoplasm, HBZ associates and inhibits GADD34 which has been demonstrated to be a mTOR inhibitor. Then, HBZ enhances mTOR activity probably for allowing its anabolic functions, though mTOR inhibits the autophagy triggering and in consequence HBZ indirectly inhibits the autophagy pathway (105, 106). This is interesting because it might be related to the strategy used by Tax to induce autophagy that is going directly to BECN1/PI3KC3 complex in a manner independent of mTOR activity status. All in all, these data demonstrate that we are not yet watching the whole panorama. Future work would shed light about the complex mechanisms in Tax-autophagy close relationship and whether it includes other autophagy related processes such as selective autophagy, non-canonical forms of autophagy, etc.

Going even further, it has been demonstrated that a constitutively activated IKK complex induces autophagy *in vitro* and *in vivo* (107). IKK is implicated in early carcinogenesis inducing autophagy in several tumors in order to cope with the stress related to tumor microenvironment (108). IKKβ seems to be crucial in this intricate mechanism since this molecule transactivates BECN1 to induce autophagy (109). With very interesting data, Peng and colleagues show that IKKβ induces accumulation of autophagosomes, but at the same time enhances the fusion of those autophagic vesicles with the MVBs, resulting in amphisomes (110). They also observed the IKKβ-mediated driving of amphisomes toward the plasma membrane with the consequent release of small extracellular vesicles (EVs) which are positive for the autophagic proteins LC3 and SQSTM-1/p62 (110). Importantly, Tax has recently been found in EVs from HTLV-1 infected T cell lines (111). Moreover, those EVs bear the viral proteins Tax and HBZ among some host proteins, and transcriptional mRNA of Tax, HBZ and 5'LTR (**Figure 1**) (111). The incubation of those EVs with uninfected cell cultures (CTLL-2 and PBMC) increases survival under stress conditions (111, 112). This was further confirmed in EVs from ATLL patients derived leukemia cells where Tax was also detected (113). In the same work, EVs purified from ATLL cell line HUT-102 were taken up by bone marrow mesenchymal stem cells (MSC) with the consequent activation of NF-κB pathway, observable morphological changes, proliferation, activation of a migratory phenotype and presence of angiogenic markers (**Figure 1**) (113). Putting together the effect of IKKβ over the autophagy pathway and the release of EVs with presence of EVs containing Tax from infected cells and

ATLL patients cells it is not difficult to speculate that both events might be connected, though it needs to be confirmed. For sure, these results broaden the views about the possible roles of Tax, and/or other HTLV-1 proteins, regarding all these pathways. Finally, we are just observing the tip of the iceberg about HTLV-1, autophagy, and their relationship in the development of ATLL.

CONCLUSIONS AND PERSPECTIVES

Most viruses have developed different strategies to overcome cell defenses over evolution, and even more, to use those cellular mechanisms for their own viral cycle. Autophagy is an important homeostatic cellular process and as such it has an antiviral program of action like virophagy and xenophagy. Indeed, HTLV-1 virus induces autophagy to foster viral production. Tax protein seems to be the wild card weapon of HTLV-1, which is able to orchestrate most of the viral action to success in its infective attempt. In the same movement, Tax engages the autophagy and the NF-κB pathways in such a way that it is enough to produce the oncogenic transformation of the cell and, indeed, go on even when Tax is not more detectable. The recent results around IKK, autophagy, the vesicular trafficking and the EVs carrying Tax let us imagine that this is just the beginning in our comprehension of this intricate process. Finally, during HTLV-1infection, Tax is in the middle of a complex crossroad that includes inflammatory signal pathways, apoptosis, autophagy, and intercellular communication, that could be the key to uncover its oncogenic transformation ability.

AUTHOR CONTRIBUTIONS

CB, DG, and MG conceived the ideas. ND, DG, CB, and MG collected literature resources. DG and MG wrote the original draft. PB, MB, and FK reviewed manuscript. ND, DP, CB, DG, and MG reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was partially supported by UBACYT-UBA-(20020190200047BA) (DLP), UBACYT-UBA-(20020190200293BA) (CB), PICT 2015-1105 - ANPCyT (MNG), PICT 2018-02220 - ANPCyT (DG), PICT 2019-00736 - ANPCyT (DP) and PICT 2019-00433 - ANPCyT (CB). The authors thank the University of Buenos Aires and the National Council for Scientific and Technological Research (CONICET) for the facilities to carry out our work every day.

REFERENCES

1. Gessain A, Cassar O. Epidemiological Aspects and World Distribution of HTLV-1 Infection. *Front Microbiol* (2012) 3:388. doi: 10.3389/fmicb.2012.00388
2. Gonçalves DU, Proietti FA, Ribas JGR, Araújo MG, Pinheiro SR, Guedes AC, et al. Epidemiology, treatment, and prevention of human T-cell leukemia virus type 1-associated diseases. *Clin Microbiol Rev* (2010) 23:577–89. doi: 10.1128/CMR.00063-09
3. Futsch N, Mahieux R, Dutartre H. HTLV-1, the Other Pathogenic Yet Neglected Human Retrovirus: From Transmission to Therapeutic Treatment. *Viruses* (2017) 10:1. doi: 10.3390/v10010001
4. Matsuoka M, Jeang K-T. Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. *Nat Rev Cancer* (2007) 7:270–80. doi: 10.1038/nrc2111
5. Chen C-J, Hsu W-L, Yang H-I, Lee M-H, Chen H-C, Chien Y-C, et al. Epidemiology of virus infection and human cancer. *Recent Results Cancer Res* (2014) 193:11–32. doi: 10.1007/978-3-642-38965-8_2
6. Tagaya Y, Matsuoka M, Gallo R. 40 years of the human T-cell leukemia virus: past, present, and future. version 1; peer review: 2 approved. *F1000Res* (2019) 8:228. doi: 10.12688/f1000research.17479.1
7. Shimoyama M. members of The Lymphoma Study Group (1984–87)*. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. *Br J Haematol* (1991) 79:428–37. doi: 10.1111/j.1365-2141.1991.tb08051.x
8. Tsukasaki K, Hermine O, Bazarbachi A, Ratner L, Ramos JC, Harrington W, et al. Definition, prognostic factors, treatment, and response criteria of adult T-cell leukemia-lymphoma: a proposal from an international consensus meeting. *J Clin Oncol* (2009) 27:453–9. doi: 10.1200/JCO.2008.18.2428
9. Bangham CRM. Human T cell leukemia virus type 1: persistence and pathogenesis. *Annu Rev Immunol* (2018) 36:43–71. doi: 10.1146/annurev-immunol-042617-053222
10. Matsuoka M, Yasunaga J. Human T-cell leukemia virus type 1: replication, proliferation and propagation by Tax and HTLV-1 bZIP factor. *Curr Opin Virol* (2013) 3:684–91. doi: 10.1016/j.coviro.2013.08.010
11. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. *Nature* (2008) 451:1069–75. doi: 10.1038/nature06639
12. Grasso D, Renna FJ, Vaccaro MI. Initial steps in mammalian autophagosome biogenesis. *Front Cell Dev Biol* (2018) 6:146. doi: 10.3389/fcell.2018.00146
13. Corona Velazquez AF, Jackson WT. So many roads: the multifaceted regulation of autophagy induction. *Mol Cell Biol* (2018) 38:1–14. doi: 10.1128/MCB.00303-18
14. Dunn WA. Studies on the mechanisms of autophagy: maturation of the autophagic vacuole. *J Cell Biol* (1990) 110:1935–45. doi: 10.1083/jcb.110.6.1935
15. Parzych KR, Klionsky DJ. An overview of autophagy: morphology, mechanism, and regulation. *Antioxid Redox Signal* (2014) 20:460–73. doi: 10.1089/ars.2013.5371
16. Fader CM, Colombo MI. Autophagy and multivesicular bodies: two closely related partners. *Cell Death Differ* (2009) 16:70–8. doi: 10.1038/cdd.2008.168
17. Peng X, Yang L, Ma Y, Li Y, Li H. Focus on the morphogenesis, fate and the role in tumor progression of multivesicular bodies. *Cell Commun Signal* (2020) 18:122. doi: 10.1186/s12964-020-00619-5
18. Buratta S, Tancini B, Sagini K, Delo F, Chiaradia E, Urbanelli L, et al. Lysosomal Exocytosis, Exosome Release and Secretory Autophagy: The Autophagic- and Endo-Lysosomal Systems Go Extracellular. *Int J Mol Sci* (2020) 21:2576. doi: 10.3390/ijms21072576
19. Kowal J, Tkach M, Théry C. Biogenesis and secretion of exosomes. *Curr Opin Cell Biol* (2014) 29:116–25. doi: 10.1016/j.ceb.2014.05.004
20. Salimi L, Akbari A, Jabbari N, Mojarad B, Vahhabi A, Szafert S, et al. Synergies in exosomes and autophagy pathways for cellular homeostasis and metastasis of tumor cells. *Cell Biosci* (2020) 10:64. doi: 10.1186/s13578-020-00426-y
21. Isola AL, Chen S. Exosomes: the messengers of health and disease. *Curr Neuropharmacol* (2017) 15:157–65. doi: 10.2174/1570159x14666160825160421
22. Anderson MR, Kashanchi F, Jacobson S. Exosomes in viral disease. *Neurotherapeutics* (2016) 13:535–46. doi: 10.1007/s13311-016-0450-6
23. Pegtel DM, Gould SJ. Exosomes. *Annu Rev Biochem* (2019) 88:487–514. doi: 10.1146/annurev-biochem-013118-111902

24. Jeppesen DK, Fenix AM, Franklin JL, Higginbotham JN, Zhang Q, Zimmerman LJ, et al. Reassessment of exosome composition. *Cell* (2019) 177:428–45.e18. doi: 10.1016/j.cell.2019.02.029
25. Savina A, Vidal M, Colombo MI. The exosome pathway in K562 cells is regulated by Rab11. *J Cell Sci* (2002) 115:2505–15.
26. Savina A, Furlán M, Vidal M, Colombo MI. Exosome release is regulated by a calcium-dependent mechanism in K562 cells. *J Biol Chem* (2003) 278:20083–90. doi: 10.1074/jbc.M301642200
27. Fader CM, Sánchez D, Furlán M, Colombo MI. Induction of autophagy promotes fusion of multivesicular bodies with autophagic vacuoles in k562 cells. *Traffic* (2008) 9:230–50. doi: 10.1111/j.1600-0854.2007.00677.x
28. Fader CM, Sánchez DG, Mestre MB, Colombo MI. TI-VAMP/VAMP7 and VAMP3/cellubrevin: two v-SNARE proteins involved in specific steps of the autophagy/multivesicular body pathways. *Biochim Biophys Acta* (2009) 1793:1901–16. doi: 10.1016/j.bbamcr.2009.09.011
29. Xu J, Camfield R, Gorski SM. The interplay between exosomes and autophagy - partners in crime. *J Cell Sci* (2018) 131:jcs215210. doi: 10.1242/jcs.215210
30. Hessvik NP, Øverbye A, Brech A, Torgersen ML, Jakobsen IS, Sandvig K, et al. PIKfyve inhibition increases exosome release and induces secretory autophagy. *Cell Mol Life Sci* (2016) 73:4717–37. doi: 10.1007/s00018-016-2309-8
31. Kudchodkar SB, Levine B. Viruses and autophagy. *Rev Med Virol* (2009) 19:359–78. doi: 10.1002/rmv.630
32. Satake M, Yamada Y, Atogami S, Yamaguchi K. The incidence of adult T-cell leukemia/lymphoma among human T-lymphotropic virus type 1 carriers in Japan. *Leuk Lymphoma* (2015) 56:1806–12. doi: 10.3109/10428194.2014.964700
33. Qayyum S, Choi JK. Adult T-cell leukemia/lymphoma. *Arch Pathol Lab Med* (2014) 138:282–6. doi: 10.5858/arpa.2012-0379-RS
34. Murphy EL, Hanchard B, Figueroa JP, Gibbs WN, Lofters WS, Campbell M, et al. Modelling the risk of adult T-cell leukemia/lymphoma in persons infected with human T-lymphotropic virus type I. *Int J Cancer* (1989) 43:250–3. doi: 10.1002/ijc.2910430214
35. Yamaguchi K. Pathogenesis of adult T-cell leukemia from clinical pathologic features. *Hum retrovirology: HTLV* (1990) 163–70.
36. Tsukasaki K, Tobinai K. Human T-cell lymphotropic virus type I-associated adult T-cell leukemia-lymphoma: new directions in clinical research. *Clin Cancer Res* (2014) 20:5217–25. doi: 10.1158/1078-0432.CCR-14-0572
37. Tsukasaki K. Adult T-cell leukemia-lymphoma. *Hematology* (2012) 17 Suppl 1:S32–5. doi: 10.1179/102453312X13336169155330
38. Rodríguez-Zúñiga MJM, Cortez-Franco F, Qujiano-Gomero E. Adult T-Cell Leukemia/Lymphoma. Review of the Literature. *Actas Dermosifiliogr* (2018) 109:399–407. doi: 10.1016/j.ad.2017.08.014
39. Bangham CRM, Toulza F. Adult T cell leukemia/lymphoma: FoxP3(+) cells and the cell-mediated immune response to HTLV-1. *Adv Cancer Res* (2011) 111:163–82. doi: 10.1016/B978-0-12-385524-4.00004-0
40. Furukawa Y, Okadome T, Tara M, Niina K, Izumo S, Osame M. Human T-cell lymphotropic virus type-I (HTLV-I)-associated myelopathy/tropical spastic paraparesis with acute type of adult T-cell leukemia. *Intern Med* (1995) 34:1130–3. doi: 10.2169/internalmedicine.34.1130
41. Furukawa Y, Kubota R, Tara M, Izumo S, Osame M. Existence of escape mutant in HTLV-I tax during the development of adult T-cell leukemia. *Blood* (2001) 97:987–93. doi: 10.1182/blood.v97.4.987
42. Ratner L, Portis T, Robek M, Harding J, Grossman W. Studies of the immortalizing activity of HTLV type 1 Tax, using an infectious molecular clone and transgenic mice. *AIDS Res Hum Retroviruses* (2000) 16:1647–51. doi: 10.1089/08892220050193092
43. Nerenberg M, Hinrichs SH, Reynolds RK, Khoury G, Jay G. The tat gene of human T-lymphotropic virus type 1 induces mesenchymal tumors in transgenic mice. *Science* (1987) 237:1324–9. doi: 10.1126/science.2888190
44. Grossman WJ, Kimata JT, Wong FH, Zutter M, Ley TJ, Ratner L. Development of leukemia in mice transgenic for the tax gene of human T-cell leukemia virus type I. *Proc Natl Acad Sci USA* (1995) 92:1057–61. doi: 10.1073/pnas.92.4.1057
45. Grassmann R, Berchtold S, Radant I, Alt M, Fleckenstein B, Sodroski JG, et al. Role of human T-cell leukemia virus type 1 X region proteins in immortalization of primary human lymphocytes in culture. *J Virol* (1992) 66:4570–5. doi: 10.1128/JVI.66.7.4570-4575.1992
46. Franchini G. Molecular mechanisms of human T-cell leukemia/lymphotropic virus type I infection. *Blood* (1995) 86:3619–39. doi: 10.1182/blood.V86.10.3619.bloodjournal86103619
47. Yoshida M. Multiple viral strategies of HTLV-1 for dysregulation of cell growth control. *Annu Rev Immunol* (2001) 19:475–96. doi: 10.1146/annurev.immunol.19.1.475
48. Wang W, Zhou J, Shi J, Zhang Y, Liu S, Liu Y, et al. Human T-cell leukemia virus type 1 Tax-deregulated autophagy pathway and c-FLIP expression contribute to resistance against death receptor-mediated apoptosis. *J Virol* (2014) 88:2786–98. doi: 10.1128/JVI.03025-13
49. Bangham CRM, Matsuoka M. Human T-cell leukaemia virus type 1: parasitism and pathogenesis. *Philos Trans R Soc Lond B Biol Sci* (2017) 372:20160272. doi: 10.1098/rstb.2016.0272
50. Karimi M, Mohammadi H, Hemmatzadeh M, Mohammadi A, Rafatpanah H, Baradaran B. Role of the HTLV-1 viral factors in the induction of apoptosis. *BioMed Pharmacother* (2017) 85:334–47. doi: 10.1016/j.biopha.2016.11.034
51. Cereseto A, Washington Parks R, Rivadeneira E, Franchini G. Limiting amounts of p27Kip1 correlates with constitutive activation of cyclin E-CDK2 complex in HTLV-I-transformed T-cells. *Oncogene* (1999) 18:2441–50. doi: 10.1038/sj.onc.1202567
52. Marriott SJ, Semmes OJ. Impact of HTLV-I Tax on cell cycle progression and the cellular DNA damage repair response. *Oncogene* (2005) 24:5986–95. doi: 10.1038/sj.onc.1208976
53. Fochi S, Mutascio S, Bertazzoni U, Zipeto D, Romanelli MG. HTLV Deregulation of the NF-κB Pathway: An Update on Tax and Antisense Proteins Role. *Front Microbiol* (2018) 9:285. doi: 10.3389/fmicb.2018.00285
54. Sun SC, Elwood J, Béraud C, Greene WC. Human T-cell leukemia virus type I Tax activation of NF-kappa B/Rel involves phosphorylation and degradation of I kappa B alpha and RelA (p65)-mediated induction of the c-rel gene. *Mol Cell Biol* (1994) 14:7377–84. doi: 10.1128/mcb.14.11.7377
55. Higuchi M, Tsubata C, Kondo R, Yoshida S, Takahashi M, Oie M, et al. Cooperation of NF-kappaB2/p100 activation and the PDZ domain binding motif signal in human T-cell leukemia virus type 1 (HTLV-1) Tax1 but not HTLV-2 Tax2 is crucial for interleukin-2-independent growth transformation of a T-cell line. *J Virol* (2007) 81:11900–7. doi: 10.1128/JVI.00532-07
56. Cheng H, Ren T, Sun S. New insight into the oncogenic mechanism of the retroviral oncoprotein Tax. *Protein Cell* (2012) 3:581–9. doi: 10.1007/s13238-012-2047-0
57. Oeckinghaus A, Ghosh S. The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harb Perspect Biol* (2009) 1:a000034. doi: 10.1101/cshperspect.a000034
58. Krappmann D, Vincendeau M. Mechanisms of NF-κB deregulation in lymphoid malignancies. *Semin Cancer Biol* (2016) 39:3–14. doi: 10.1016/j.semcancer.2016.05.002
59. Sun S-C. The noncanonical NF-κB pathway. *Immunol Rev* (2012) 246:125–40. doi: 10.1111/j.1600-065X.2011.01088.x
60. Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. *Cell* (2008) 132:344–62. doi: 10.1016/j.cell.2008.01.020
61. Israël A. The IKK complex, a central regulator of NF-kappaB activation. *Cold Spring Harb Perspect Biol* (2010) 2:a000158. doi: 10.1101/cshperspect.a000158
62. Liu F, Xia Y, Parker AS, Verma IM. IKK biology. *Immunol Rev* (2012) 246:239–53. doi: 10.1111/j.1600-065X.2012.01107.x
63. Zhang F, Yang L, Li Y. The role of A20 in the pathogenesis of lymphocytic malignancy. *Cancer Cell Int* (2012) 12:44. doi: 10.1186/1475-2867-12-44
64. Massoumi R. CYLD: a deubiquitination enzyme with multiple roles in cancer. *Future Oncol* (2011) 7:285–97. doi: 10.2217/fon.10.187
65. Sarkar B, Nishikata I, Nakahata S, Ichikawa T, Shiraga T, Saha HR, et al. Degradation of p47 by autophagy contributes to CADM1 overexpression in ATLL cells through the activation of NF-κB. *Sci Rep* (2019) 9:3491. doi: 10.1038/s41598-019-39424-7
66. Sun SC, Ballard DW. Persistent activation of NF-kappaB by the tax transforming protein of HTLV-1: hijacking cellular IkappaB kinases. *Oncogene* (1999) 18:6948–58. doi: 10.1038/sj.onc.1203220
67. Pise-Masison CA, Choi KS, Radonovich M, Dittmer J, Kim SJ, Brady JN. Inhibition of p53 transactivation function by the human T-cell lymphotropic

virus type 1 Tax protein. *J Virol* (1998) 72:1165–70. doi: 10.1128/JVI.72.2.1165-1170.1998
68. Yin MJ, Christerson LB, Yamamoto Y, Kwak YT, Xu S, Mercurio F, et al. HTLV-I Tax protein binds to MEKK1 to stimulate IkappaB kinase activity and NF-kappaB activation. *Cell* (1998) 93:875–84. doi: 10.1016/s0092-8674(00)81447-6
69. Wu X, Sun S-C. Retroviral oncoprotein Tax deregulates NF-kappaB by activating Tak1 and mediating the physical association of Tak1-IKK. *EMBO Rep* (2007) 8:510–5. doi: 10.1038/sj.embor.7400931
70. Nejmeddine M, Barnard AL, Tanaka Y, Taylor GP, Bangham CRM. Human T-lymphotropic virus, type 1, tax protein triggers microtubule reorientation in the virological synapse. *J Biol Chem* (2005) 280:29653–60. doi: 10.1074/jbc.M502639200
71. Harhaj NS, Sun S-C, Harhaj EW. Activation of NF-kappa B by the human T cell leukemia virus type I Tax oncoprotein is associated with ubiquitin-dependent relocalization of I kappa B kinase. *J Biol Chem* (2007) 282:4185–92. doi: 10.1074/jbc.M611031200
72. Lamsoul I, Lodewick J, Lebrun S, Brasseur R, Burny A, Gaynor RB, et al. Exclusive ubiquitination and sumoylation on overlapping lysine residues mediate NF-kappaB activation by the human T-cell leukemia virus tax oncoprotein. *Mol Cell Biol* (2005) 25:10391–406. doi: 10.1128/MCB.25.23.10391-10406.2005
73. Huang J, Ren T, Guan H, Jiang Y, Cheng H. HTLV-1 Tax is a critical lipid raft modulator that hijacks IkappaB kinases to the microdomains for persistent activation of NF-kappaB. *J Biol Chem* (2009) 284:6208–17. doi: 10.1074/jbc.M806390200
74. Harhaj EW, Sun SC. IKKgamma serves as a docking subunit of the IkappaB kinase (IKK) and mediates interaction of IKK with the human T-cell leukemia virus Tax protein. *J Biol Chem* (1999) 274:22911–4. doi: 10.1074/jbc.274.33.22911
75. Li ZW, Chu W, Hu Y, Delhase M, Deerinck T, Ellisman M, et al. The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. *J Exp Med* (1999) 189:1839–45. doi: 10.1084/jem.189.11.1839
76. Jin DY, Giordano V, Kibler KV, Nakano H, Jeang KT. Role of adapter function in oncoprotein-mediated activation of NF-kappaB. Human T-cell leukemia virus type I Tax interacts directly with IkappaB kinase gamma. *J Biol Chem* (1999) 274:17402–5. doi: 10.1074/jbc.274.25.17402
77. Mori N, Fujii M, Ikeda S, Yamada Y, Tomonaga M, Ballard DW, et al. Constitutive Activation of NF-κB in Primary Adult T-Cell Leukemia Cells. *Blood* (1999) 93:2360–8. doi: 10.1097/00042560-199904010-00027
78. Béraud C, Sun SC, Ganchi P, Ballard DW, Greene WC. Human T-cell leukemia virus type I Tax associates with and is negatively regulated by the NF-kappa B2 p100 gene product: implications for viral latency. *Mol Cell Biol* (1994) 14:1374–82. doi: 10.1128/mcb.14.2.1374
79. Uhlik M, Good L, Xiao G, Harhaj EW, Zandi E, Karin M, et al. NF-kappaB-inducing kinase and IkappaB kinase participate in human T-cell leukemia virus I Tax-mediated NF-kappaB activation. *J Biol Chem* (1998) 273:21132–6. doi: 10.1074/jbc.273.33.21132
80. Xiao G, Harhaj EW, Sun SC. NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. *Mol Cell* (2001) 7:401–9. doi: 10.1016/s1097-2765(01)00187-3
81. Harhaj EW, Giam C-Z. NF-κB signaling mechanisms in HTLV-1-induced adult T-cell leukemia/lymphoma. *FEBS J* (2018) 285:3324–36. doi: 10.1111/febs.14492
82. Fochi S, Ciminale V, Trabetti E, Bertazzoni U, D'Agostino DM, Zipeto D, et al. NF-κB and MicroRNA Deregulation Mediated by HTLV-1 Tax and HBZ. *Pathogens* (2019) 8:290. doi: 10.3390/pathogens8040290
83. Ren T, Takahashi Y, Liu X, Loughran TP, Sun SC, Wang HG, et al. HTLV-1 Tax deregulates autophagy by recruiting autophagic molecules into lipid raft microdomains. *Oncogene* (2015) 34:334–45. doi: 10.1038/onc.2013.552
84. Levine B. Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. *Cell* (2005) 120:159–62. doi: 10.1016/j.cell.2005.01.005
85. Lee J-S, Li Q, Lee J-Y, Lee S-H, Jeong JH, Lee H-R, et al. FLIP-mediated autophagy regulation in cell death control. *Nat Cell Biol* (2009) 11:1355–62. doi: 10.1038/ncb1980
86. Orvedahl A, Sumpter R, Xiao G, Ng A, Zou Z, Tang Y, et al. Image-based genome-wide siRNA screen identifies selective autophagy factors. *Nature* (2011) 480:113–7. doi: 10.1038/nature10546
87. Mao J, Lin E, He L, Yu J, Tan P, Zhou Y. Autophagy and viral infection. *Adv Exp Med Biol* (2019) 1209:55–78. doi: 10.1007/978-981-15-0606-2_5
88. Blanchet FP, Moris A, Nikolic DS, Lehmann M, Cardinaud S, Stalder R, et al. Human immunodeficiency virus-1 inhibition of immunoamphisomes in dendritic cells impairs early innate and adaptive immune responses. *Immunity* (2010) 32:654–69. doi: 10.1016/j.immuni.2010.04.011
89. Orvedahl A, Alexander D, Tallóczy Z, Sun Q, Wei Y, Zhang W, et al. HSV-1 ICP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. *Cell Host Microbe* (2007) 1:23–35. doi: 10.1016/j.chom.2006.12.001
90. Dreux M, Chisari FV. Autophagy proteins promote hepatitis C virus replication. *Autophagy* (2009) 5:1224–5. doi: 10.4161/auto.5.8.10219
91. Shrivastava S, Raychoudhuri A, Steele R, Ray R, Ray RB. Knockdown of autophagy enhances the innate immune response in hepatitis C virus-infected hepatocytes. *Hepatology* (2011) 53:406–14. doi: 10.1002/hep.24073
92. Tang S-W, Chen C-Y, Klase Z, Zane L, Jeang K-T. The cellular autophagy pathway modulates human T-cell leukemia virus type 1 replication. *J Virol* (2013) 87:1699–707. doi: 10.1128/JVI.02147-12
93. Wang J, Niu Z, Shi Y, Gao C, Wang X, Han J, et al. Bcl-3, induced by Tax and HTLV-1, inhibits NF-κB activation and promotes autophagy. *Cell Signal* (2013) 25:2797–804. doi: 10.1016/j.cellsig.2013.09.010
94. Takahashi Y, Coppola D, Matsushita N, Cualing HD, Sun M, Sato Y, et al. Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. *Nat Cell Biol* (2007) 9:1142–51. doi: 10.1038/ncb1634
95. Ohashi Y, Tremel S, Williams RL. VPS34 complexes from a structural perspective. *J Lipid Res* (2019) 60:229–41. doi: 10.1194/jlr.R089490
96. Chen L, Liu D, Zhang Y, Zhang H, Cheng H. The autophagy molecule Beclin 1 maintains persistent activity of NF-κB and Stat3 in HTLV-1-transformed T lymphocytes. *Biochem Biophys Res Commun* (2015) 465:739–45. doi: 10.1016/j.bbrc.2015.08.070
97. Shibata Y, Oyama M, Kozuka-Hata H, Han X, Tanaka Y, Gohda J, et al. p47 negatively regulates IKK activation by inducing the lysosomal degradation of polyubiquitinated NEMO. *Nat Commun* (2012) 3:1061. doi: 10.1038/ncomms2068
98. Hartmann-Petersen R, Wallace M, Hofmann K, Koch G, Johnsen AH, Hendil KB, et al. The Ubx2 and Ubx3 cofactors direct Cdc48 activity to proteolytic and nonproteolytic ubiquitin-dependent processes. *Curr Biol* (2004) 14:824–8. doi: 10.1016/j.cub.2004.04.029
99. Wójcik C, Yano M, DeMartino GN. RNA interference of valosin-containing protein (VCP/p97) reveals multiple cellular roles linked to ubiquitin/proteasome-dependent proteolysis. *J Cell Sci* (2004) 117:281–92. doi: 10.1242/jcs.00841
100. Pujari R, Hunte R, Khan WN, Shembade N. A20-mediated negative regulation of canonical NF-κB signaling pathway. *Immunol Res* (2013) 57:166–71. doi: 10.1007/s12026-013-8463-2
101. Schwob A, Teruel E, Dubuisson L, Lormières F, Verlhac P, Abudu YP, et al. SQSTM-1/p62 potentiates HTLV-1 Tax-mediated NF-κB activation through its ubiquitin binding function. *Sci Rep* (2019) 9:16014. doi: 10.1038/s41598-019-52408-x
102. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun J-A, Outzen H, et al. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem* (2007) 282:24131–45. doi: 10.1074/jbc.M702824200
103. Svenning S, Johansen T. Selective autophagy. *Essays Biochem* (2013) 55:79–92. doi: 10.1042/bse0550079
104. Journo C, Filipe J, About F, Chevalier SA, Afonso PV, Brady JN, et al. NRP/Optineurin Cooperates with TAX1BP1 to potentiate the activation of NF-kappaB by human T-lymphotropic virus type 1 tax protein. *PloS Pathog* (2009) 5:e1000521. doi: 10.1371/journal.ppat.1000521
105. Mukai R, Ohshima T. HTLV-1 HBZ positively regulates the mTOR signaling pathway via inhibition of GADD34 activity in the cytoplasm. *Oncogene* (2014) 33:2317–28. doi: 10.1038/onc.2013.181
106. Vescovo T, Pagni B, Piacentini M, Fimia GM, Antonioli M. Regulation of autophagy in cells infected with oncogenic human viruses and its impact on cancer development. *Front Cell Dev Biol* (2020) 8:47. doi: 10.3389/fcell.2020.00047
107. Criollo A, Senovilla L, Authier H, Maiuri MC, Morselli E, Vitale I, et al. The IKK complex contributes to the induction of autophagy. *EMBO J* (2010) 29:619–31. doi: 10.1038/emboj.2009.364

108. Leonardi M, Perna E, Tronnolone S, Colecchia D, Chiariello M. Activated kinase screening identifies the IKBKE oncogene as a positive regulator of autophagy. *Autophagy* (2019) 15:312–26. doi: 10.1080/15548627.2018.1517855
109. Dikic I, Elazar Z. Mechanism and medical implications of mammalian autophagy. *Nat Rev Mol Cell Biol* (2018) 19:349–64. doi: 10.1038/s41580-018-0003-4
110. Peng X, Yang L, Ma Y, Li X, Yang S, Li Y, et al. IKKβ activation promotes amphisome formation and extracellular vesicle secretion in tumor cells. *Biochim Biophys Acta Mol Cell Res* (2021) 1868:118857. doi: 10.1016/j.bbamcr.2020.118857
111. Jaworski E, Narayanan A, Van Duyne R, Shabbeer-Meyering S, Iordanskiy S, Saifuddin M, et al. Human T-lymphotropic virus type 1-infected cells secrete exosomes that contain Tax protein. *J Biol Chem* (2014) 289:22284–305. doi: 10.1074/jbc.M114.549659
112. Otaguiri KK, Dos Santos DF, Slavov SN, Depieri LV, Palma PVB, Meirelles FV, et al. TAX-mRNA-Carrying Exosomes from Human T Cell Lymphotropic Virus Type 1-Infected Cells Can Induce Interferon-Gamma Production In Vitro. *AIDS Res Hum Retroviruses* (2018) 34:1075–82. doi: 10.1089/AID.2018.0115
113. El-Saghir J, Nassar F, Tawil N, El-Sabban M. ATL-derived exosomes modulate mesenchymal stem cells: potential role in leukemia progression. *Retrovirology* (2016) 13:73. doi: 10.1186/s12977-016-0307-4

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Ducasa, Grasso, Benencio, Papademetrio, Biglione, Kashanchi, Berini and Garcia. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Oncology

REVIEW
published: 15 April 2021
doi: 10.3389/fonc.2021.637594

Autophagy Process in Trophoblast Cells Invasion and Differentiation: Similitude and Differences With Cancer Cells

Lorena Carvajal[1], *Jaime Gutiérrez*[2], *Eugenia Morselli*[1,3]* *and Andrea Leiva*[2]*

[1] Department of Physiology, Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, Santiago, Chile, [2] School of Medical Technology, Health Sciences Faculty, Universidad San Sebastian, Santiago, Chile, [3] Autophagy Research Center, Santiago, Chile

Early human placental development begins with blastocyst implantation, then the trophoblast differentiates and originates the cells required for a proper fetal nutrition and placental implantation. Among them, extravillous trophoblast corresponds to a non-proliferating trophoblast highly invasive that allows the vascular remodeling which is essential for appropriate placental perfusion and to maintain the adequate fetal growth. This process involves different placental cell types as well as molecules that allow cell growth, cellular adhesion, tissular remodeling, and immune tolerance. Remarkably, some of the cellular processes required for proper placentation are common between placental and cancer cells to finally support tumor growth. Indeed, as in placentation trophoblasts invade and migrate, cancer cells invade and migrate to promote tumor metastasis. However, while these processes respond to a controlled program in trophoblasts, in cancer cells this regulation is lost. Interestingly, it has been shown that autophagy, a process responsible for the degradation of damaged proteins and organelles to maintain cellular homeostasis, is required for invasion of trophoblast cells and for vascular remodeling during placentation. In cancer cells, autophagy has a dual role, as it has been shown both as tumor promoter and inhibitor, depending on the stage and tumor considered. In this review, we summarized the similarities and differences between trophoblast cell invasion and cancer cell metastasis specifically evaluating the role of autophagy in both processes.

Keywords: autophagy, placentation, trophoblast and cancer cells, cellular proliferation, migration and invasion, vasculogenic capacity, vascular remodeling, immune evasion

OPEN ACCESS

Edited by:
Benjamin Kidder, Wayne State University, United States

Reviewed by:
Xiaoyin Lu, University of Texas MD Anderson Cancer Center, United States
Hua Zhang, First Affiliated Hospital of Chongqing Medical University, China

***Correspondence:**
Andrea Leiva
andrea.leiva@uss.cl
Eugenia Morselli
emorselli@bio.puc.cl

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *03 December 2020*
Accepted: *08 March 2021*
Published: *15 April 2021*

Citation:
Carvajal L, Gutiérrez J, Morselli E and Leiva A (2021) Autophagy Process in Trophoblast Cells Invasion and Differentiation: Similitude and Differences With Cancer Cells. Front. Oncol. 11:637594. doi: 10.3389/fonc.2021.637594

INTRODUCTION

The placentation is a complex process that involves different stages, which quickly and efficiently leads to the development of the placenta, a temporary organ. The placenta is developed through regulated and dynamic cellular processes that include embryo pre-implantation and implantation, decidua formation, trophoblast proliferation, trophoblast differentiation into the invasive

phenotype, and vascular remodeling (1). Interestingly, during placentation, the ability of trophoblast cells to proliferate, invade, and evade the immune system, resemble those induced by cancer cells during tumor growth (2). Indeed, the processes of proliferation, migration, and invasion in cancer cells and trophoblast derived cells share different molecules such as growth factors, cell adhesion molecules, surface receptors, matrix-digesting enzymes, and enzymes inhibitors, proto-oncogenes, hormones, and peptides, among others (3). These molecules regulate different processes that are highly controlled in trophoblasts, with trophoblast-derived cells following an organized pattern without metastasizing to new tissues, while the same pathways are dysregulated in cancer, driving metastasis (4).

In addition to sharing proliferative and invasive features, trophoblasts and cancer cells, actively modulate the host immune response to develop and sustain nutrient supply (5). Interestingly, it has been described that activation of autophagy occurs in both processes, regulating placental and cancer development (6, 7). However, how autophagy modulation affects trophoblast function is not entirely known (8). Consistently, the role of autophagy in cancer development is still a matter of study due to its dual role in tumor onset and progression (9, 10). Indeed, the role of autophagy in tumor development is controversial and dependent on tumor stage and type. It has been suggested that autophagy could promote aggressive characteristics of cancer cells, such as increased cellular invasion (11, 12), but also be a barrier to cancer proliferation (13–15). Additionally, autophagy also provides the microenvironment for placentation and cancer growth. This review will summarize the parallels between trophoblast-derived cells in placentation and cancer cells in tumor growth and metastasis with a final focus on the role of autophagy in both processes.

DEVELOPMENT OF THE HUMAN PLACENTA

The placenta is a temporary organ that maintains and protects the fetus during pregnancy controlling the maternal-fetal exchange of nutrients, gases, and metabolic waste. Human pregnancy begins with the physiological preparation of the endometrium modulated by hormones such as progesterone and estrogen, which regulate growth factors, cytokines, and adhesion molecules that allow the blastocyst's implantation (16).

The placenta develops from the trophectoderm (TE), the outer layer of the blastocyst from which derives the undifferentiated cytotrophoblast (CTB). The CTB originates two main villus structures: the floating villus, where CTBs fuse to form the multinuclear syncyiotrophoblast (STB) and the anchoring villus (17–20) (**Figure 1**). The STB acts as an exchange barrier with the maternal blood to assure nutrients as well as waste and gases exchange with the fetal blood (21). The floating villus cells proliferate to form primary villi, which show further branching, forming the intervillous space. The branching to secondary and tertiary villi, allows the expansion of the STB surface area, which favors an efficient nutrient exchange with the fetal blood (19).

As mentioned, the second villus structure derived from the CTB is the anchoring villus, whose main function is to mediate the placental attachment to the endometrium in the uterine wall, to sustain fetal growth (17–20) (**Figure 1**). In the distal tip of the anchoring villus it is possible to find a group of proliferating cells known as cell column trophoblast (CCT). From these cells emerge the placental giant trophoblast (PGT) that mediates the early histotrophic nutrition of the embryo and of extravillous trophoblast cells (EVT). EVTs are a group of non-proliferating trophoblast cells characterized by a highly invasive phenotype. They invade the maternal decidua and the first third of the myometrium, playing a crucial role in the histotrophic nutrition of the fetus, immunomodulation and remodeling of the uterine spiral arteries (**Figure 1**) (19).

These activities are mediated by specialized subgroups of EVTs, characterized by specific markers: the endovascular EVT (evEVT) and the interstitial EVT (iEVT) (**Table 1**). The evEVTs migrate through the lumen of the spiral arteries forming a trophoblast plug reducing the maternal blood flow towards the intervillous space during the early stages of placenta development, permitting histiotrophic nutrition (46). Additionally, evEVT maintain the oxygen concentration low, which is required for placental development and successful trophoblast differentiation and may also promote favorable trophoblast migration and endothelial cell replacement, both required for vascular remodeling. Finally, at the end of first trimester the trophoblast plug formed by evEVT is disintegrated (47, 48).

The main function of iEVT is to participate to the immune tolerance and placental invasion of maternal tissues. iEVTs express Human Leukocyte antigen-G (HLA-G), a nonclassical major histocompatibility complex (MHC) (**Table 1**), which is essential to modulate the immune tolerance at the maternal-fetal interface by regulating the interaction and communication with the Uterine Natural Killers (uNK) (49).

iEVTs invade trough the interstitium of the decidua and myometrium towards the maternal spiral arteries (46). Once there, the iEVT acquires an endothelial-like phenotype under conditions of low oxygen concentration (approximately 8% oxygen) replacing the maternal endothelial cells of the uterine spiral arteries. It is important to highlight that many studies refer to this low oxygen concentration as an "hypoxic" environment. However, the reduced oxygen level corresponds to the physiological level required for placenta development, which is maintained until the end of the first trimester (below 20 mmHg). Thus, as this occurs in normal placental development, we will refer to this low oxygen condition as normoxic and not hypoxia (50–52).

The process of invasion by EVTs allows the replacement of the endothelial layer of the maternal spiral arteries, which is

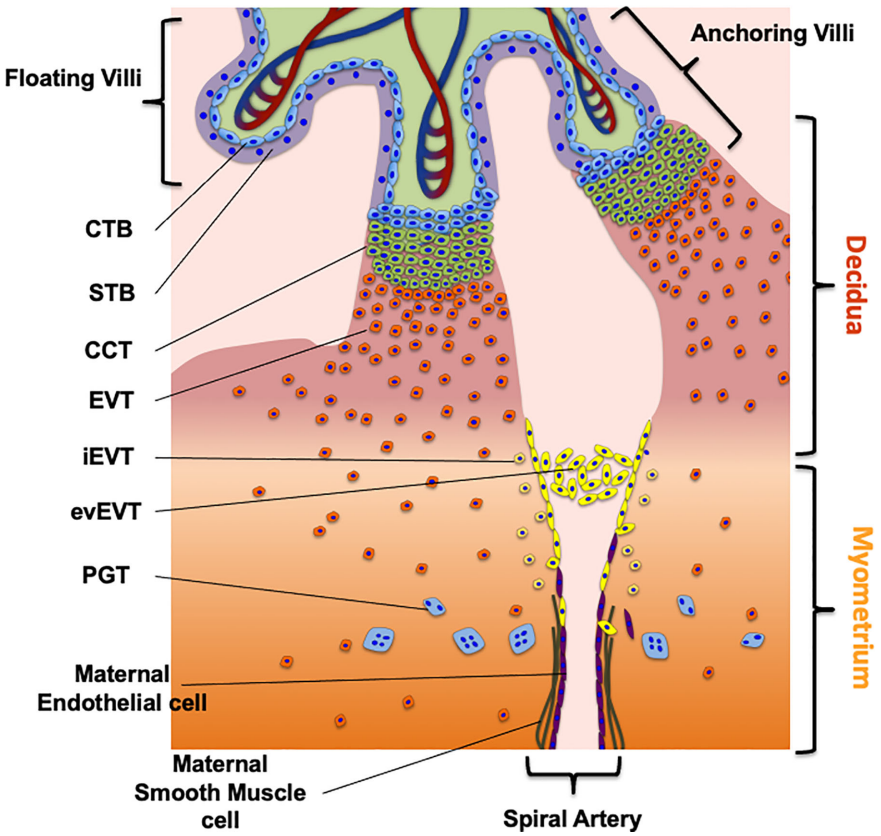


FIGURE 1 | The maternal fetal interface and trophoblast cells subtypes. The figure shows the placental cell types required for the early first trimester human placentation as well as the route to migrate and invade the decidua and myometrium. The different trophoblast subtypes are villous cytotrophoblast (CTB), synctiotrophoblast (STB), cell column trophoblast (CCT), extravillous trophoblast (EVT), endovascular EVT (evEVT), interstitial EVT (iEVT), placental giant trophoblast (PGT). The complete description of the process is in the section "*Development of the Human Placenta*" of the review.

essential for an appropriate placental perfusion that maintains an adequate fetal growth. This complex process not only involves different placental cell types, but also a wide range of molecules related to cell growth (i.e., hormones and growth factors), cellular adhesion (i.e., Integrins, Cadherins), tissular remodeling (i.e., Metalloproteases) and proteins related to immune tolerance (**Table 1**) (19, 53). In addition to the classical markers, other molecules including structural and adhesion related proteins, proteins associated to invasion, immunity, embryonic stem cell-associated transcription regulators and oncogenes have been recently described as markers of CTB, STB, iEVT or eEVT (54–58).

CTB and CCT but not EVT cells show proliferative activity and generate cells that stop the proliferation and start to differentiate (20). The process of proliferation and differentiation of CTB into migratory, invasive EVT and endothelial-like trophoblast shows similarities with the process of tumor formation and metastasis of cancer cells. The main similarities are: (i) tissue invasion, (ii) immune system modulation and (iii) vascularization. Despite this, a crucial difference between trophoblast derived and cancer cells is that while in trophoblast cells these processes are regulated, this regulation is lost in cancer cells (4).

The physiological process of placentation responds to a controlled program that results in changes in gene expression and cell cycle. As such, when placentation is not kept under control, malformation of the placenta, pregnancy pathologies and abortions can occur (17, 20, 59). Different abnormal placentation processes have been described, which are characterized by abnormal trophoblast invasion such as abnormally invasive placentas. Abnormal placentation processes are: (i) placenta accreta (abnormal adherence with direct contact to myometrium), (ii) placenta increta (placental villi penetrate into the myometrium) and (iii) percreta (placental villi penetrate trough myometrium to uterine serosa and into the surrounding structures such as the bladder). It has been suggested that trophoblast cells of this abnormally invasive placentas lose their physiological regulation, leading to increased proliferative activity during the invasion, behaving like cancer cells (60, 61).

TABLE 1 | Classical markers of trophoblast-derived cells in the human placenta and its expression in vasculogenic mimicry on human cancer.

Type of marker	Marker	CTB	STB	CCT	evEVT	iEVT	Reference	Expression in vasculogenic mimicry on human cancer	Reference
Epithelial marker	CK7	+	+	+	NA	+	(22)	–	
Mesenchymal marker	Vimentin	–	–	–	NA	–	(23)	Hepatocellular, Colorectal, Ovarian, pancreatic, Large lung cancer, Non-small cell lung cancer, renal cell carcinoma	(24–26)
Integrins									
	α1 β1	–	–	–	+	+	(27)	–	
	α5 β1	–	NA	–	NA	+	(27)	Glioblastoma, melanoma	(26, 28, 29)
	α6 β4	+	–	+	NA	–	(27)	–	
	αv β5	+	–	+	–	–	(30)	–	
	αv β3	–	–	–	+	+	(30)	Breast cancer, prostate, colon, melanoma	(31)
Cell adhesion molecules									
	VE-Cadherin	–	–	–	+	+	(32)	Melanoma, hepatocellular, Non-small cell lung cancer, colorectal, prostate, large-cell lung cancer, gastric	(26, 33),
	E-cadherin	+	+	+	–	–	(32)	Ovarian, colorectal, pancreatic, large-cell lung cancer, hepatocellular, Non-small cell lung cancer, melanoma	(26, 34),
	PECAM	+	+	+	+	–	(32)	Melanoma	(31)
	NCAM	–	–	–	+	–	(32, 35),	–	
Metalloproteases									
	MMP-2	NA	NA	+	NA	+	(36)	Melanoma, ovarian cancer	(34, 37),
	MMP-9	NA	NA	+	NA	+	(36)	Ovarian cancer, Hepatocellular	(26, 34),
	MMP-14 (MT1-MMP)	+	+	+	–	+	(38–40)	Melanoma	(37)
Hormones									
	hCG α	+	+	+/-	+/-	+/-	(41)	–	
	hCG β	+/-	+	–	–	–	(41)	–	
	hPL	–	+	+	+	NA	(27)	–	
Growth factors									
	TGF β	+	+	+	NA	–	(42)	Hepatocellular	(26)
	VEGF	NA	+	NA	NA	+	(43)	Ovarian cancer	(34)
	sFLT-1	NA	+	NA	NA	+	(43)	–	
	Endoglin	–	+	+	NA	–	(44)	–	
Immune factors									
	HLA-G	–	–	–	NA	+	(23, 45),	–	

Classical markers of trophoblast-derived cells in the human placenta and their expression in malign tumors are showed in the table. CK7, cytokeratin 7; VE-cadherin, Vascular endothelial-Cadherin; PECAM, platelet endothelial cell adhesion molecule; NCAM, neural cell adhesion molecule; MMP-2, matrix metallopeptidase 2; MMP-9, matrix metallopeptidase 9; MMP-14, matrix metallopeptidase 14; hCG α, human chorionic gonadotropin α; hCG β, human chorionic gonadotropin β; hPL, human placental lactogen; TGF β, transforming growth factor beta; VEGF, vascular endothelial growth factor; sFLT-1, Fms-like tyrosine kinase-1; EGF, epidermal growth factor; HLA-G, human leukocyte antigen G.

SIMILARITIES AND DIFFERENCES BETWEEN CANCER CELLS AND TROPHOBLAST DERIVED CELLS

Cellular Proliferation, Migration, and Invasion

Both cancer cells and trophoblast derived cells express different molecules such as growth factors, proto-oncogenes, enzymes, cell surface receptors, enzyme receptors, hormones and peptides, whose activation mediates their high proliferative, migratory and invasive capacity. During placentation, growth factors such as Epidermal Growth Factor (EGF), Hepatocyte Growth Factor (HGF), Vascular Endothelial Growth Factor (VEGF), Placental Growth Factor (PLGF), Insulin-like Growth Factor (IGF), Transforming Growth Factor (TGF) and their corresponding receptors are among the main factors that regulate CTB proliferation, acting in a paracrine and autocrine manner (62). These growth factors bind to Tyrosine Kinase Receptors to activate the Mitogen-activated Protein/extracellular Signal-regulated kinase/Extracellular Signal-Regulated Kinase (MEK/ERK) proliferation pathway and the Phosphatidylinositol 3-kinase/Protein Kinase B (PI3K/Akt) anti-apoptosis pathway (3). Moreover, proto-oncogenes play an essential role in the etiology of cancer inducing its growth. As cancer cells, trophoblast derived cells express several proto-oncogenes; for example, CTB and STB exclusively express proto-oncogenes that encode Growth Factor Receptor c-erbB1 (Human Epidermal Growth Factor Receptor 1 (HER1), Epidermal Growth Factor Receptor 1 (ERBB1 or EGF-receptor)) (63). Also, trophoblast cells such as CTB, STB, and EVT encode for a Receptor Tyrosine Kinase (RTK), namely c-erbB2 (HER2/neu, ERBB2), c-fms (CSF1R), c-met (MET) and c-kit (KIT) (64–67), as well as for transcription factors that have been implicated in trophoblast invasion such as c-fos (FOS) and c-jun (JUN), in addition to c-myc (MYC) and c-ets1 (ETS) (68–71). Additionally, in iEVT, *c-sis* (SIS, Platelet-derived Growth Factor Beta (PDGFB)) is expressed, which encodes for one of the two chains (the B-chains) constituting Platelet-derived

Growth Factor (PDGF) (72) and in EVT the *c-ras* family (Kirsten rat Sarcoma viral oncogene (K-RAS), Neuroblastoma RAS viral oncogene homolog (N-RAS), and Harvey rat sarcoma (H-RAS)) is expressed encoding for Rat sarcoma (RAS) proteins that regulate cellular proliferation and inflammation in the human placenta (73, 74). All the aforementioned proto-oncogenes are crucial in the first step of malignant transformation and its physiological expression occurs during the first week of pregnancy promoting proliferation, migration, and invasion of the trophoblast (2).

The Telomerase is a factor that regulates the proliferative capacity of a cell, as it maintains chromosome stability in actively dividing cells (75). CTB expresses a functional Telomerase, which is downregulated during differentiation, but expressed in term placenta. During human pregnancy, Telomerase activity is the highest during the first trimester, and decreases with the maturation of the placenta (76). Telomerase activity ensures a high rate of proliferation and could be a factor controlling placental growth (77–79). Consistently, in cancer cells, the Telomerase allows uncontrolled cell proliferation, which is essential for tumor progression (80). Additionally, Survivin, a protein overexpressed in many cancers (81), where it promotes proliferation and inhibits apoptosis, is expressed in trophoblast cells, however its role in this location has not been elucidated yet (82, 83). Altogether these studies indicate that the Telomerase and Survivin have an important role in cell proliferation in both trophoblast and cancer cells.

As mentioned, placental development during the first trimester occurs in a stable state of low oxygen concentration (84); by comparison, in tumors, hypoxia is necessary to support tumor growth and metastasis (85). In response to low oxygen levels, cells upregulate Hypoxia-Inducible Factor (HIF), a family of transcription factors that functions as a Heterodimer with a regulatory α subunit (HIF-α) and a constitutive β subunit (HIF-β) (86, 87). The activation of the different HIF isoforms leads to the transcription of genes involved in several processes such as metabolism, angiogenesis, and immunomodulation (86). Thus, this low oxygen concentration environment in trophoblast and cancer cells could be considered as key to stimulate proliferation, invasion, and vasculogenesis in host tissues (88).

During placentation and cancer growth, invasion is required to provide blood and nutrient supply. Different events need to occur for a successful invasion process: (i) changes in the expression of Cell Adhesion molecules (ii) secretion of Proteases, and (iii) availability of Growth Factors (5). One feature shared by both cell types is the process of epithelial to mesenchymal transition (EMT), which leads to the loss of cell-to-cell contact inhibition, and to the increased expression of proteins that degrade the extracellular matrix. During EMT the Integrin expression pattern changes, and the expression of E-cadherin decreases, enhancing cell movement through tissues by reducing cell polarity (89, 90).

EVT and invasive cancer cells also share enzymes required for the degradation of the basal membrane that allow the process of invasion. Among those there are Serine Proteases, Cathepsins and Matrix Metalloproteinases (MMPs), the Heparan Sulfate-degrading Endoglycosidase, the Protease-Activated Receptor (PAR) and the Receptor of Thrombin (91, 92). These enzymes are expressed transiently in the trophoblast, in a very regulated manner, while in cancer cells their expression becomes constitutive (2, 4). As an example, the expression of MMP-2 and MMP-9 is increased during trophoblast invasion, promoting proteolysis and therefore invasion. Importantly, when the invasion is completed, decidual cells inhibit MMP-2 and MMP-9 activity by the release of protease inhibitors (53, 93, 94). When the control of the protease activity is lost abnormally invasive placentas develop, consistently, this regulation disappears in cancer invasive cells (60).

Additionally, Placenta-Specific Protein 8 (PLAC-8) is a placental protein implicated in embryo implantation, which is expressed in iEVT on the feto-maternal interface promoting trophoblast invasion and migration (57, 95) nevertheless PLAC-8 is also expressed in cells from different cancers such as lung adenocarcinoma, pancreatic cancer, colorectal cancer, gastrointestinal cancer, and cervical cancer (96–100), where it is involved in malignant tumor progression by regulating cell differentiation (100), proliferation (101), apoptosis (102) and autophagy by mediating autophagosome/autolysosome fusion (103).

In conclusion, for the physiological invasion of iEVT and for the pathological metastasis of cancer cells similar mechanisms are used. However, despite the similarities between them, they show a key difference: while the trophoblast follows an organized pattern of proliferation, differentiation and invasion without metastasizing to new tissues; cancer cells spread through the host tissue with a high proliferation rate, with the final objective of being able to metastasize to other tissues (3, 5).

Vasculogenic Capacity

The vascularization capacity is also a common feature between trophoblast and cancer cells as an abundant blood supply is necessary both for the growth of the tumor nodule, and for the implanting embryo. To date, three processes of vessel growth have been described: vasculogenesis, angiogenesis and vascular mimicry (104, 105). Vasculogenesis is the process of new blood vessel formation from angioblast precursor cells; angiogenesis is the process of growth and development of new capillary blood vessels from pre-existing vessels like new branches; vascular mimicry corresponds to vessel growth from adult cells into a vascular-like phenotype (105, 106).

During the first trimester of pregnancy, vasculogenesis and angiogenesis are consecutive processes. Mesenchymal stem cells differentiate to become hemangiogenic stem cells, then, in a paracrine manner, the CTB induces the formation of the first vessels *via* induction of VEGF signaling. After that, the existing vessels become longer, a process mediated by VEGF and PLGF (107). In cancer, angiogenesis is crucial for the newly formed tumor nodule, since it provides blood continuously to initiate progression and tumor growth (108). This process involves molecular and cellular interactions between cancerous cells, endothelial cells, and some components of the Extra-Cellular Matrix (ECM), such as matrix proteins (Fibronectin, Laminin, Collagen), receptors (Integrins) and enzymes that degrade the

ECM [MMP and Tissue Inhibitor of Metalloproteinase (TIMP)]. Specific proteins such as VEGF and FGF are secreted by cancer cells to stimulate the proliferation of capillary endothelial cells leading to the sprout and branching of them through the ECM (109). Recent evidence suggests that in tumors resistant to different anti-angiogenic drugs, in addition to angiogenesis, other processes that contribute to tumoral vascularization occur, namely vasculogenesis and vascular mimicry (110, 111).

Interestingly, human EVT and invasive cancer cells have similar patterns of integrins expression (**Table 1**), which allows the EVT to adopt a vascular phenotype capable of invading maternal spiral arterioles, a process similar to what occurs in endothelial cells when they migrate towards the tumor (5). This turnover of endothelial cells to form new vessels requires different angiogenesis regulators that are similar between EVT and cancer cells (3). Among those, VEGF and PLGF promote angiogenesis and are regulated by hypoxia and Fibroblast Growth Factor (FGF) can initiate angiogenesis in both cell types. Conversely, Angiostatin, Fibronectin, and Tissue inhibitor of Metalloproteinases act as angiogenesis inhibitors (109, 112, 113).

Additionally, both cell types are able to directly contribute to their own blood supply by inducing vascular mimicry (88), enhancing gene expression patterns and signaling pathways shared by the two cell types (5). As an example, the Galactose-binding protein Galectin-3, which is known to provide a vascular phenotype, is highly expressed in EVT (114) and is also a key factor for the development of aggressive melanomas (115).

In summary, the process of angiogenesis is essential both in trophoblast and cancer cells. In cancer it drives tumor growth and metastasis, and in pregnancy it allows proper embryo implantation and placentation. However, while trophoblast cells create new blood vessels inducing a controlled process of vasculogenesis, the angiogenesis in cancer is uncontrolled (3).

Immune Evasion

For proper development, trophoblast and cancer cells evade the immune response of the host. During placentation, for the development of the maternal-fetal interface, the maternal decidua basalis, where the maternal immune cells are located, interacts with the fetal derived placental iEVT. Additionally the placenta produces anti-inflammatory Cytokines, TGF-β2, Interleukin (IL)-4 and IL-10, which reduce the deleterious effects of pro-inflammatory cytokines (4). Fas Ligand (Fas-L) expression on trophoblast promotes apoptosis of Fas-expressing lymphocytes of maternal origin, having a role in placental invasion during implantation (116). The position of trophoblast cells in the placenta encasing the embryo produces a barrier between maternal and fetal cells, finally being the placenta the main separation of fetal and maternal blood and lymphatic systems, preventing the immune system of the mother to perceive fetal antigens. During the first trimester the immune cells located in the decidua basalis are Natural Killer (NK, 70%), Macrophages (20-25%) and T Lymphocytes [3-10%, (117–119)]. It has been suggested that the presence of progesterone and TGF-β1 in the decidua promotes the differentiation of these NK into mature Uterine Natural Killers (uNK) (120, 121). uNK cells are more immunomodulatory than cytotoxic, they secrete Growth factors, Angiogenic factors and Cytokines facilitating immune tolerance which suggests uNK play a role in implantation, invasion and vascular remodeling of spiral artery remodeling, regulating EVT invasion (122) by a mechanism that has not yet been totally clarified (49, 123, 124). Additionally, macrophages have been shown as capable of regulating the process of spiral artery remodeling, metabolic regulation of lipids, tissue regeneration, inflammation and fetal antigen recognition (125). Furthermore, they can influence EVT function as they are more abundant at the invasive front and implantation site (126, 127). Despite these studies, their role in placentation, as support of trophoblast cells, has not been fully elucidated. The role of T lymphocytes is also poorly understood, however, it has been described that they could have a role in controlling infections caused by bacteria located at the maternal-fetal interface (119).

In cancer, NK cells are known to contribute to tumor development *via* secretion of Cytokines (128, 129). Additionally, cancer cells express tumor-associated Macrophages, which can have an inflammatory and immunosuppressive role, being key in tumor progression and metastasis (130). Fas-mediated apoptosis and the expression of Fas-L allow many cancers to attack the immune system (131, 132). Regulatory T cells are implicated in mediating tolerance in cancer and pregnancy; immunophenotypically expressing Cluster of differentiation (CD); CD4, CD25 and Forkead Box P3 (FOXP3) (133). In pregnancy, regulatory T cells are induced by paternal/fetal alloantigens (134), which is crucial for maternal-fetal tolerance. In cancer, regulatory T cells are implicated in impaired antitumor immunity, suppression of effector T lymphocytes proliferation, and increased tumor blood vessel density, suggesting an essential link between immunity and angiogenesis (5). iEVT express Human leukocyte antigen- G (HLA-G) (19), which suppresses cytolytic killing by NK and cytotoxic T cells inducing apoptosis of immune cells (49). HLA-G regulates cytokine production in blood mononuclear cells, reducing stimulatory capacity and impairing the maturation of dendritic cells (5). In tumors, HLA-G promotes immune evasion by interacting with NK cells *via* Inhibitory receptors and Killer cell Immunoglobulin-like receptor (KIR) (135). This molecule can directly mediate immune tolerance by inhibiting receptors, predominantly Immunoglobulin-like Transcript (ILT) 2 and 4 expressed on immune effectors (136). Finally, HLA-G has been detected in melanoma and solid tumors including cervical cancer, gastrointestinal cancer and breast cancer (137–139).

In conclusion, both trophoblast and cancer cells actively modulate the host immune response by different mechanisms that are induced by similar cells and molecules, finally promoting cell invasion.

AUTOPHAGY

Autophagy is a catabolic process highly conserved among eukaryotic organisms, which allows the lysosomal-mediated degradation of cytoplasmic components, thus contributing to

cell homeostasis. Three types of autophagy have been described based on the mechanism by which the cargo is delivered to the lysosome: (i) microautophagy, where the cytosolic material is delivered to the lysosome by a direct invagination or protrusion of the lysosomal membrane (140) (ii) chaperone-mediated autophagy, where unfolded soluble proteins containing a specific consensus motif translocate across the lysosomal membrane (141–143), and macroautophagy, herein referred to as autophagy, where the cargo is sequestered in a special double membrane organelle known as autophagosome and then delivered to the lysosome. Briefly, during autophagy the autophagosome fuses to lysosome, forming the autolysosome, where the cargo is degraded (144) (**Figure 2**). The new metabolites derived from the degradation return then back to the cytosol and will be used for the synthesis of new macromolecules and/or energy production (145). Different autophagy-related (ATG) proteins are required for autophagy to occur, these are organized in protein complexes that are necessary in the different steps of the autophagic process. These can be divided into five stages (initiation, nucleation, elongation, fusion with the lysosome, and cargo degradation) (**Figure 2**). During "initiation" the unc-51-like kinase 1 (ULK1)/focal adhesion kinase family interacting protein of 200 kDa (FIP200)/ATG13 complex (ULK1 complex) is activated, in response to the metabolic status of the cell (146). Once active, the ULK1 complex translocates to membranous sites, known as omegasomes, where the autophagosome will form (i.e. endoplasmic reticulum and mitochondria contact sites) (147, 148). Then, during the "nucleation", the isolation membrane of the new autophagosome is generated. This process is mediated by the kinase complex formed by Vacuolar Sorting Protein (VPS) 34 (VPS34), Beclin-1, and VPS15 and Autophagy related 14-like protein (ATGL14), which generates phosphatidylinositol 3-phosphate (PI3P), necessary for the recruitment of the machinery required for the generation of the new autophagosome (146, 149). ATG9-containing vesicles cycle between the omegasome and the Golgi/endosomes, and they contribute to the recruitment of membranes for the nucleation of the phagophore (147, 150, 151). Then, the phagophore extends during the "elongation stage", a process that is tightly regulated by two ubiquitin-like systems: the microtubule-associated protein 1A/1B-light chain 3 (MAP1LC3A, also known as LC3-I) system and the ATG5–ATG12 system (152) and by the ATG5–ATG12 complex. The ATG5–ATG12 complex then interacts with ATG16L, forming a new complex that works like an E3 enzyme, assisting the incorporation of LC3-II into the membrane of the phagophore (153). In parallel with the elongation the autophagic cargo is selected. Proteins targeted for autophagy are labeled with the receptor p62/Sequestosome-1 (p62/SQSTM1), which interacts with LC3 through an LC3 interacting region (LIR) (154, 155). Following elongation, the elongated phagophore is finally closed forming the autophagosome. This step is completed by a membrane abscission process mediated by the endosomal-sorting complex

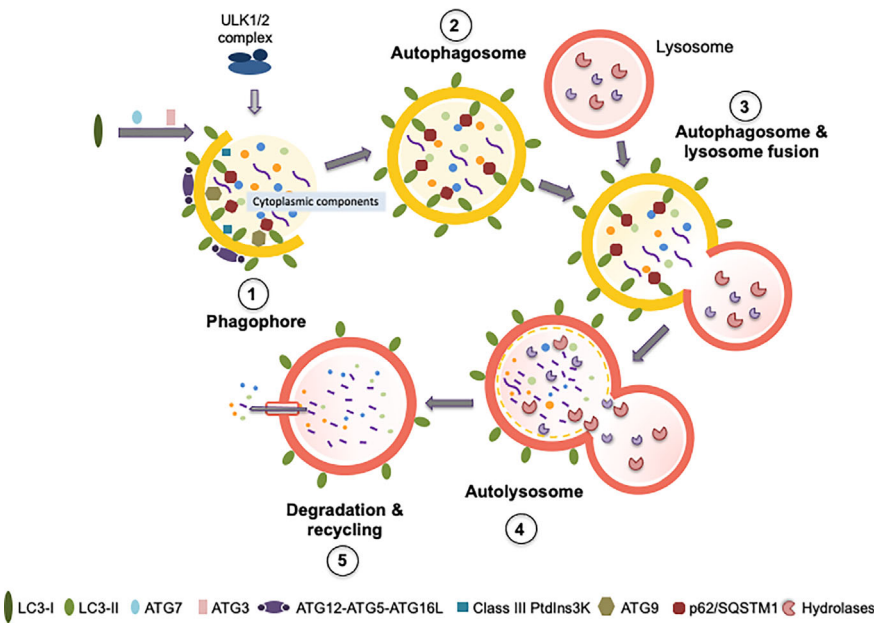


FIGURE 2 | Autophagy process and principal proteins involved in the different steps. The figure shows the principal proteins required for autophagy process, the different steps of the process are described in the section "*Autophagy*" (initiation, nucleation, elongation, fusion with the lysosome, and cargo degradation and recycling). Figure (1) corresponds to phagophore formation that includes initiation and nucleation. (2) Autophagosome maturation includes the elongation process. (3) Autophagosome and lysosome fusion. (4) Represent the structure of the autolysosome, and (5) corresponds to degradation and recycling. In each step are indicated the main proteins required: ULK1/2 complex, LC3 I, LC3II, ATG7, ATG3, ATG12-ATG5-ATG16L, Class III PtdIns3K, ATG9 and p62/SQSTM1. The yellow semi-circumferences and circumferences correspond to the phagosome membrane. See the main text for further details.

required for transport (ESCRT) (156, 157). Upon closure, the nascent autophagosome dissociates from the assembly site and undergoes maturation (158). The mature autophagosome then fuses with the lysosome generating autolysosomes (159), a process mediated by Rab GTPases, membrane-tethering complexes and soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (160). The inner membrane of the autolysosome breaks down and the process of autophagosomal cargo degradation begins (161). The degradation products are recycled and turn back to the cytosol for being reused (162, 163) (**Figure 2**).

Autophagy is a complex and highly regulated process that under stress conditions such as hypoxia, low glucose concentration and oxidative stress is triggered to promote cell survival or leads to cell death. Physiologically autophagy maintains cellular and energy homeostasis, cooperates with the immune system to promote adaptation, and represents a quality control system for proteins and organelles (164). Impaired autophagy contributes to the development of neurodegenerative (165), infectious (166) and metabolic diseases (167, 168), due to the accumulation of abnormal and damaged proteins and/or organelles, forming intracellular aggregates that induce cellular stress, finally promoting cell death.

During placentation it has been reported that autophagy could be relevant for different processes required for a proper development of the placenta; however, how this occurs is still under investigation (7). On the other hand, in the context of cancer, autophagy has a dual role, where it could be tumor-suppressive or tumor-promoting depending on the stage of cancer development and the type of cancer considered (10).

ROLE OF AUTOPHAGY IN IMMUNE EVASION AND VASCULAR REMODELING: DIFFERENCES BETWEEN PLACENTATION AND CANCER

As previously described, the processes of placentation and tumor development share similarities and autophagy activation has been described in both (6, 7). The role of autophagy in cancer has been widely explored, however, as previously mentioned, its role has not completely been elucidated (169). On the other hand, how modulation of autophagy affects trophoblast function is still largely unknown.

Role of Autophagy in Placental and Cancer Immune Evasion

The role of autophagy in the placentation process remains unclear, and its contribution to immune evasion is still unknown (170). It has been described that autophagy is highly activated in decidualized endometrium of early pregnancy, which increases NK cell adhesion and retention in the decidua. Also, when autophagy is inhibited, decidual NK (dNK) cell residence is decreased, contributing to spontaneous abortion (171). Tan et al. described that autophagy levels are highly reduced in cases of recurrent miscarriage. Indeed, suppression of autophagy in an *in vitro* model of trophoblast cells enhances the cytotoxicity activity of dNK, impairing trophoblast invasion, finally causing abortion (172).

On the other hand, autophagy has been describes as an important regulator of cancer immunity in the tumor microenvironment; however, the exact mechanisms involved remain unclear (173, 174). The tumor microenvironment contains different factors that promote autophagy, such as hypoxia or inflammation (166, 175). Remarkably, it has been described that autophagic activation correlates with immune evasion (176, 177). Conversely, inhibition of autophagy associates with NK-dependent immune responses. In breast cancers, in tumors presenting hypoxia, blocking autophagy restores NK-mediated lysis *in vitro*, facilitating breast tumor elimination by NK cells in mice (178). Inhibition of autophagy also reduces NK cell-mediated cytotoxicity in melanoma (175), non-small cell lung cancer (179) and liver cancer (180). In contrast, the role of autophagy is dual in the response to immune cell recognition, being a suppressor or inductor of tumorigenesis depending on the specific context (181). Altogether these data suggest that autophagy actively participates and regulates the immune evasion of dNK in placental development and NK activity in cancer cells. However, the mechanism involved in both phenomena remains to be elucidated, a crucial aspect that needs to be studied for the development of immunotherapy in each field.

Role of Autophagy in Placental Vascular Remodeling

As indicated, trophoblast invasion and vascular remodeling allows the replacement of the endothelial layer of the maternal spiral arteries, which is essential for proper placental perfusion and adequate fetal growth. It has been described that activation of autophagy occurs in human placentas from normal pregnancies at weeks 8 to 12 of gestation, as indicated by LC3 and Beclin-1 protein in CTB and STB cells (182) (**Table 2**). Moreover, autophagosomes have been identified in human placentas throughout gestation from early (8 weeks) (189, 194) to term pregnancies (39 weeks) (189, 194, 261).

The key role of autophagy in implantation was demonstrated by studies in ATG5-deficient mouse oocytes, where pre-implantation cannot occur correctly. Indeed, autophagy increases in the oocytes after fertilization, and it is necessary for pre-implantation development, which is essential to allow the differentiation from zygote to blastocyst in mammals (262). In different mouse models it has been shown that proteins of the LC3 family are expressed in the labyrinth zone and in the decidua basalis, which suggests a possible role in the placentation process (263).

In vitro assays in the trophoblast cell line HTR8/SVneo (i.e., a first-trimester human trophoblast cell line) showed increased LC3 lipidation and LC3 puncta in cells cultured in 2% of oxygen, which mimics the physiological O_2 concentration in the early pregnancy period (186, 189, 192). In the same cell line, higher LC3 and Beclin-1 expression was determined in conditions of

TABLE 2 | Changes in protein involved in the autophagic process described in human placental tissues and trophoblast cell lines.

Study model Human placental tissue	Cell line	Autophagy marker	Reference
CS vs VD	–	↑LC3	(183)
PES vs N	–	↑LC3, ↑Beclin-1	(184)
IUGR vs N	–	↑LC3, ↑Beclin-1	(185)
–	HTR-8/SVneo inhibition of Hypoxia inducible factor (HIF)-1α	↑LC3, ↓Beclin-1	(186)
CTB exposed to hypoxia vs normoxia	–	↑LC3, ↑p62	(187)
MC sIUGR vs MC	–	↑LC3	(188)
EVT exposed to hypoxia vs normoxia	HTR-8/SVneo exposed to hypoxia vs normoxia	↑LC3, ↓p62	(189)
FTP, N	–	LC3, Beclin-1	(182)
SP vs IL	–	↑LC3	(190)
NE vs N	–	↑LC3	(191)
–	HTR-8/SVneo exposed to Cobalt chloride ($CoCl_2$)	↑LC3	(192)
PE vs N	JEG-3	↑LC3	(193)
FTP,MD, N, CS, VD	–	= LC3, = Beclin-1	(194)
N		↑LC3, ↑ATG5-12	(195)
	JEG-3	↑LC3	
PIH vs N	–	↑LC3, ↓p62	(196)
PE vs N HUVEC	HTR-8/SVneo	↑LC3, ↑Beclin-1	(197)
Early placenta-SM vs Normal-early placenta	–	↑LC3	(198)
–	JEG-3 with ASAH1 inhibition or ceramide treatment	↑LC3, ↑p62	(199)
Fetal membrane SP vs Fetal membrane N	–	↓Beclin-1, ↓ ATG3, ↓ATG5, ↓ATG7, ↓ATG12, ↓AT16L1	(200)
–	BeWo treated with dexamethasone	↑LC3	(201)
PE vs N	–	↑p62	(202)
STB treated with punicalagin	–	↓ LC3, ↓p62	(203)
OB vs N	–	↑Beclin-1, ↑ATG3, ↑ATG7, ↑LC3	(204)
–	BeWo exposed to an increase in reactive oxygen species	↑ATG5, ↑ATG7, ↑LC3, ↓p62	(205)
PE vs N	JEG-3	↓LC3, ↓Beclin-1	(206)
PTD vs N		↓LC3, ↑p62, ↓ ATG6L, = Beclin-1, = ATG7	(207)
	BeWo	↑LC3	
PES vs N	HTR-8/SVneo, JEG-3	↑LC3, ↑ATG4B	(208)
PTD with/without inflammatory lesions	–	↑LC3	(209)
EOPE vs N	–	↑LC3	(210)
GDM vs N	–	↓Beclin-1, ↑LC3, ↑p62	(211)
FGR vs N	–	↑LC3, ↑ Beclin-1, ↓ p62	(212)
GDM vs N		↑LC3, ↓ p62	(213)
	HTR-8/SVneo	↑LC3, ↓ p62, ↑ATG5	
FTP vs N	–	↓LC3	(214)
IUGR, EOPE vs N	–	↑LC3, ↑ Beclin-1	(215)
Placenta with Malaria vs N	–	↑LC3, = ATG4B, = p62	(216)
–	BeWo exposed to overexpression of CYP11A1 gene	↑LC3, ↑ Beclin-1	(217)
IUGR vs N	–	↑LC3, ↓ p62	(218)
PE vs N	–	↑Beclin-1, ↑p62	(219)
–	JEG-3 exposed to cigarette smoke	↑LC3, ↑p62	(220)
FTP Primary trophoblast	BeWo	↑LC3, ↑p62	(221)
–	Sw.71 exposed to saturated fatty acids	↑p62, ↑LC3	(222)
–	BeWo exposed to cobalt and chromium nanoparticles	↑LC3, ↑p62	(223)
PE vs N	–	↑LC3, ↑p62	(224)
PES vs N	–	↑LC3, ↓ p62	(225)
CTB	–	↑LC3, ↑ Beclin-1	(226)
ICP vs N	HTR-8/SVneo	↑LC3, ↑ATG5, ↑ ATG7, ↑Beclin-1	(227)
–	HTR-8/SVneo exposed to Titanium dioxide nanoparticles	↑LC3, ↑p62	(228)
Early miscarriage vs N	–	↑ LC3, ↑ATG5, ↑Beclin-1	(229)
–	HTR-8/SVneo exposed to Titanium dioxide nanoparticles	↑LC3, ↑p62	(230)
–	HTR-8/SVneo, JEG-3 associated to long noncoding RNA H19 downregulation	↑LC3, ↑Beclin-1, ↓ p62	(231)
CTB	BeWo	↑ATG16, ↑ATG5-ATG12, ↑ATG7, ↑LC3, ↓p62	(232)
–	HchEpC1b, HTR-8/SVneo exposed to platinum nanoparticles (npt)	↑LC3, ↓ p62	(233)
HDCP vs N	HPVEC	↓Beclin-1,↓LC3	(234)
Placenta accrete vs N	–	↑LC3, ↑Beclin-1, ↑p62	(235)

(Continued)

TABLE 2 | Continued

Study model Human placental tissue	Cell line	Autophagy marker	Reference
–	HchEpC1b exposed to oxidative stress	↑p62,	(236)
–	JEG-3 exposed to Cadmium	↑LC3, ↑p62	(237)
CTB	BeWo	↑LC3	(238)
Plasmodium falciparum-infected women vs non infected	–	↓Beclin-1,↓LC3	(239)
–	HTR-8/SVneo with inhibition of Death-associated protein kinase-3	↑LC3, ↑p62,↑ATG5	(240)
–	HTR-8/SVneo exposed to hypoxia	↑LC3	(241)
–	HTR-8/SVneo exposed to oxidative stress	↓Beclin-1,↓LC3, ↑p62	(242)
–	HTR-8/SVneo with knockdown of plasmacytoma variant translocation 1	↓Beclin-1,↓LC3, ↑p62	(243)
–	HTR-8/SVneo exposed to Hydrogen peroxide	↑LC3, ↑Beclin-1	(244)
GDM vs N CTB	–	↓Beclin-1, ↓ATG5, ↓LC3, ↓p62	(245)
Anemic vs polycythemic territories in TAPS	–	↑LC3, ↑p62	(246)
–	BeWo under hyperglycemic conditions reduce	↓LC3, ↓p62	(247)
With vs without Probiotic supplementation in SP	–	↓Beclin-1	(248)
OB vs N	–	↓LC3	(249)
–	HTR-8/SVneo with overexpression of homeobox protein A7	↑LC3, ↓p62	(250)
PE vs N	–	↑LC3	(251)
–	HTR-8/SVneo exposed to high glucose	↑LC3, ↓p62	(252)
GDM vs N	–	↑LC3, ↑ATG7	(253)
DMSC from EPSM vs DSC normal pregnancy	–	↑P62, ↓ LC3, ↓ATG5	(171)
–	JAr exposed to cyclopamine and/or Gant61	↑LC3	(254)
With vs without mycophenolic Acid treatment in DMSC normal pregnancy	–	↑LC3, ↓p62	(255)
FGR vs N	BeWo	↑LC3, ↑Beclin-1	(256)
N exposed to hypoxia	BeWo exposed to hypoxia	↑LC3, ↑p62	(257)
–	HTR-8/SVneo exposed to α-solanine	↑LC3, ↑Beclin-1↑ATG13, = p62	(258)
PE vs N	–	↑p62, = LC3	(259)
–	HTR-8/SVneo and Jar with Placenta specific 8 (PLAC8) overexpression	↑ATG5-ATG12, ↑Beclin-1, ↓LC3	(260)

Changes in protein involved in the autophagic process described in human placental tissues and trophoblast cell lines are showed in the table. Human placental tissue abbreviations: N, normal term pregnancy; FTP, first trimester placenta; MD, midgestation; CS, cesarean section; VD, vaginal delivery; PE, preeclampsia; PES, severe preeclampsia; IUGR, intrauterine growth restriction; FGR, fetal growth restriction; CTB, cell primary culture from human placenta cytotrophoblasts; STB, cell primary culture from human placenta syncytiotrophoblasts; MC, monochorionic twin; MC sIUGR, monochorionic twin with selective intrauterine growth restriction; EVT, cell primary culture first trimester extravillous trophoblast; SP, spontaneous labor; IL, induced labor; NE, neonatal encephalopathy; EOPE, early-onset preeclampsia; HUVEC, human umbilical vein endothelial cells; PIH, pregnancy-induced hypertension; EPSM, spontaneous miscarriage; OB, maternal obesity; PTD, preterm delivery; GDM, gestational diabetes mellitus; HDCP, hypertensive disorder complicating pregnancy; ICP, intrahepatic cholestasis of pregnancy; TAPS, monochorionic twin anemia-polycythemia sequence; DMSC, decidua mesenchymal stromal cells from human placenta. Cell line abbreviations: HTR-8/SVneo, human first-trimester extravillous trophoblast cell line; JEG-3, human choriocarcinoma JEG-3 cell line; BeWo, human placental choriocarcinoma cell line; Sw.71, human first trimester trophoblast cell line; HPVEC, human placental microvascular endothelial cells; HchEpC1b, extravillous trophoblast cell line; JAr, human choriocarcinoma trophoblast cell line.

enhanced oxidative stress (264). Additionally, using a model of autophagy-deficient EVT cells (cells expressing a ATG4B-negative mutant), the relevance of autophagy in the trophoblast in the process of invasion was shown, as the process was impaired in autophagy-deficient cells (189). Consistently, in a mouse model where the ATG7 gene was deleted only in trophoblast (not in fetuses), the placentas were smaller than in wild type, due to reduced trophoblast invasion and low vascular remodeling. Remarkably, this result needs to be compared with those described in cancer cells lacking ATG7, which is described in the next section. Altogether, these studies demonstrate that autophagy plays a key role in trophoblast function, especially in invasion and vascular remodeling during placentation (**Table 2**). Despite this, how modulation of autophagy affects trophoblast function in pathological conditions has not been elucidated.

Importantly, even if previous research indicates a positive correlation between autophagy and cell invasion and vice-versa (192, 264), the role of the whole autophagic process, intended as autophagic flux, defined as the whole process from autophagosome formation up to its fusion with lysosome and cargo degradation (7), in the development of pregnancy-associated diseases such as preeclampsia (PE), gestational diabetes, or fetal growth (FGR) is still controversial. Indeed, for example, in homogenized tissue from PE placenta and in trophoblast cells obtained from PE placentas has been described that LC3 and Beclin-1 are increased (197, 215), suggesting that increased markers of autophagy correlate with a poor placentation process (187). However, another study in PE placenta and in the cell line JEG-3 showed an increase in LC3 without changes in Beclin-1 (265). Furthermore, additional work showed, in homogenized tissue from PE placentas, a decrease in

LC3 and Beclin-1 (206) and an increase in Beclin-1 and p62/SQSTM1 (219). These controversial results could be due to different factors: (i) the placenta is a complex organ, with different cell types that perform different functions, so it is not appropriate to use placenta homogenates and evaluate autophagy in these samples, as the levels of autophagy can be different in the different cell types. (ii) The time at which the analysis is performed is important. Indeed, as reported, it has been described that autophagy plays different roles in embryogenesis and implantation, while its role in the later stages of pregnancy is still unknown (8). (iii) It is key to evaluate a set of autophagic markers to study the autophagic flux to reach a conclusion (at least LC3 and an autophagic receptor such as p62/SQSTM1), unfortunately, some of the studies only evaluate a single autophagic protein, which is not sufficient to clearly indicate what is happening in autophagy but only suggest that the condition reported might affect this cellular process (**Table 2**). Thus, the available information related with the role of autophagy in placentation in terms of specific cells involved, cellular processes affected beyond migration of invasion (i.e., processes of differentiation to endothelial phenotype, angiogenesis, vasculogenesis or immune control) and the modulation of autophagy according to gestational age, as well as the complete autophagic flux in these different processes still needs to be elucidated.

Role of Autophagy in Vascular Remodeling in Cancer

As mentioned, the role of autophagy in tumor development is controversial and dependent of the tumor characteristics and stage of tumor development (266). Briefly, it has been suggested that autophagy could promote aggressive characteristics of cancer cells such as increased cellular invasion (11, 12), but it also represents a barrier for cancer proliferation (13–15).

In cancer cells, the inhibition of autophagy results in impaired metabolism proliferation, survival, and spontaneous tumor malignancy depending not only on the tumor type but also of its temporal development (267). This has been demonstrated in different types of cancers using genetically engineered mouse models with ablation of ATGs and consequently autophagy. For instance, in pancreatic ductal adenocarcinoma, loss of ATG5 increases tumor initiation but avoids invasive cancer progression (268). Consistently, in prostate cancer, lack of ATG7 delayed tumor cell proliferation (269) and in lung cancer driven by oncogenic Kras, the deletion of ATG7 reduces cell proliferation and tumor weight compared with mice with intact ATG7 (270). Conversely, Rao et al. show that ATG5 deletion accelerates early oncogenesis, increasing the number of tumor foci and the transition from hyperplasia to adenomas; however as cancer develops, lack of ATG5 reduces the progression from adenoma to adenocarcinoma, resulting in a decrease of tumors mass and enhanced lifespan in mice (271). Altogether, these studies demonstrate that autophagy plays a crucial role in cancer cells.

According to the stage, during the early phases of solid tumor formation, autophagy plays an anti-tumorigenic (272) effect because it limits the production of DNA damaging agents [i.e. Reactive Oxygen Species (ROS)], it promotes the elimination of oncogenic proteins, and stimulates the induction of the immune response in response to cellular stress (273). Additionally, it has been shown that autophagy could promote senescence in tumor cells in response to oncogenic stress, which results in decreased tumor growth (274, 275). On the other hand, it has been described that during tumor progression, autophagy increases the tolerance to stressful conditions such as metabolic changes and hypoxia within the tumor microenvironment, leading to enhanced tumor cell survival and playing a pro-tumorigenic role (276, 277). Autophagy can also increase metastasis, supporting tumor growth, interacting with pathways involved in cell motility and invasion (6), including the promotion of Focal Adhesion (FA) turnover, which is a component of the cell migration machinery, being Paxillin the essential FA protein degraded by autophagy (278) and ECM proteins. For example, in pancreatic ductal adenocarcinomas hypoxia induces autophagy resulting in degradation of Lumican, an extracellular matrix protein highly upregulated in different cancers (279). Autophagy is also enhanced upon oncogenic RAS activation (280, 281) and is required for the production of multiple secreted factors, which include IL-6 and MMP2 in tumors bearing RAS mutations, facilitating cancer cell invasion (282). All the described data indicate that cancer cell migration could be molecularly regulated by autophagy and vice versa, providing metabolites and nutrients in stress conditions to the different cell types that form the tumor microenvironment (169).

Thus, autophagy has a dual role in cancer since in tumor initiation limits DNA damage agents such as ROS and increases tumor cell senescence leading to an anti-tumorigenic environment, preventing tumor promotion. However, in established tumors autophagy provides the necessary conditions for tumors to growth, regulating the invasion and migration process enhancing tumor cell survival increasing resistance to stressful conditions (176). Something similar occurs in trophoblast cells, where it has been suggested that autophagy regulates invasion, migration and vascular remodeling of trophoblasts, allowing the optimal development of the placenta (7). One difference between both processes is that autophagy has a role in the promotion of the placentation process from fertilization, whereas, as mentioned above, at the beginning of tumor development autophagy exerts anticarcinogenic functions protecting the host tissue, but as the tumor progresses, autophagy supports tumor metastasis, enhancing tumor cell survival by increasing the resistance to stressful conditions (283). Finally, the role of autophagy in cancer cells and trophoblast derived cells appears quite similar, since it provides the conditions to carry out cellular functions depending on the timing or stage in cancer, promoting or stopping tumor growth, while in the trophoblast autophagy could favor optimal placentation. Nevertheless, the precise role of autophagy in modulating the described cellular processes involved in vascular remodeling in cancer progression or placentation needs to be fully studied.

CONCLUSION

In conclusion, the physiological placentation process of trophoblast and the pathological metastasis of cancer cells share similar mechanisms to proliferate, migrate, and invade both trophoblast and cancer cells, modulating host immune response. However, the main difference is that trophoblast follows an organized pattern without metastasizing new tissues. On the other hand, another shared process is autophagy, which is required for invasion of trophoblast, and it has been shown in cancer has a dual role being a tumor promoter and inhibitor, depending on the stage and tumor considered. Nevertheless, the precise role of autophagy in cancer progression or placentation needs to be thoroughly studied. These studies could give a new insight in cancer biology by evaluating the similarities with trophoblast cells and the highly regulated behavior they have in placentation.

AUTHOR CONTRIBUTIONS

LC and JG: review of the literature. AL and EM: preparation of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT 1180935 JG, 1200499 EM, 1190250 AL). LC holds ANID (Chile) fellowship.

REFERENCES

1. Turco MY, Moffett A. Development of the human placenta. *Development* (2019) 146(22):dev163428. doi: 10.1242/dev.163428
2. Ferretti C, Bruni L, Dangles-Marie V, Pecking AP, Bellet D. Molecular circuits shared by placental and cancer cells, and their implications in the proliferative, invasive and migratory capacities of trophoblasts. *Hum Reprod Update* (2007) 13(2):121–41. doi: 10.1093/humupd/dml048
3. West RC, Bouma GJ, Winger QA. *Shifting perspectives from "oncogenic" to oncofetal proteins; how these factors drive placental development*. *Reprod Biol Endocrinol* (2018) 16(1):1–12. doi: 10.1186/s12958-018-0421-3
4. Soundararajan R, Rao AJ. Trophoblast 'pseudo-tumorigenesis': Significance and contributory factors. *Reprod Biol Endocrinol* (2004) 2:15. doi: 10.1186/1477-7827-2-15
5. Holtan SG, Creedon DJ, Haluska P, Markovic SN. Cancer and pregnancy: Parallels in growth, invasion, and immune modulation and implications for cancer therapeutic agents. *Mayo Clin Proc* (2009) 84(11):985–1000. doi: 10.4065/84.11.985
6. Mowers EE, Sharifi MN, Macleod KF. Functions of autophagy in the tumor microenvironment and cancer metastasis. *FEBS J* (2018) 285(10):1751–66. doi: 10.1111/febs.14388
7. Nakashima A, Tsuda S, Kusabiraki T, Aoki A, Ushijima A, Shima T, et al. Current understanding of autophagy in pregnancy. *Int J Mol Sci* (2019) 20 (9):1–13. doi: 10.3390/ijms20092342
8. Nakashima A, Aoki A, Saito S. The Role of Autophagy in Maintaining Pregnancy. *Autophagy Curr Trends Cell Physiol Pathol* (2016) 329. doi: 10.5772/63475
9. Grandér D, Panaretakis T. Autophagy: cancer therapy's friend or foe? *Future Med Chem* (2010) 2(2):285–97. doi: 10.4155/fmc.09.155
10. Saha S, Panigrahi DP, Patil S, Bhutia SK. Autophagy in health and disease: A comprehensive review. *Biomed Pharmacother* (2018) 104:485–95. doi: 10.1016/j.biopha.2018.05.007
11. Gao L, Wang Q, Ren W, Zheng J, Li S, Dou Z, et al. The RBP1–CKAP4 axis activates oncogenic autophagy and promotes cancer progression in oral squamous cell carcinoma. *Cell Death Dis* (2020) 11(6):1–16. doi: 10.1038/s41419-020-2693-8
12. Li X, Gao L, Zheng L, Shi J, Ma J. BMP4-mediated autophagy is involved in the metastasis of hepatocellular carcinoma via JNK/Beclin1 signaling. *Am J Transl Res* (2020) 12(6):3068–77.
13. Sudsaward S, Khunchai S, Thepmalee C, Othman A, Limjindaporn T, Yenchitsomanus PT, et al. Endoplasmic reticulum stress, unfolded protein response and autophagy contribute to resistance to glucocorticoid treatment in human acute lymphoblastic leukaemia cells. *Int J Oncol* (2020) 57(3):835–44. doi: 10.3892/ijo.2020.5089
14. Wu L, Wang Z, Li X, He X, Han Y, Chen Y, et al. Autophagy mediates bronchial cell malignant transformation induced by chronic arsenic exposure via MEK/ERK1/2 pathway. *Toxicol Lett* (2020) 332:155–63. doi: 10.1016/j.toxlet.2020.06.006
15. Jiao YJ, Dan ZD, Cui B, Zhang C, Wei TF, Chang S, et al. Disruption of the EGFR-SQSTM1 interaction by a stapled peptide suppresses lung cancer via activating autophagy and inhibiting EGFR signaling. *Cancer Lett* (2020) 474:23–35. doi: 10.1016/j.canlet.2020.01.004
16. Cross JC, Werb Z, Fisher SJ. Implantation and the placenta: Key pieces of the development puzzle. *Science* (1994) 266(5190):1508–18. doi: 10.1126/science.7985020
17. Gude NM, Roberts CT, Kalionis B, King RG. Growth and function of the normal human placenta. *Thromb Res* (2004) 114(5–6):397–407. doi: 10.1016/j.thromres.2004.06.038
18. Wang Y, Zhao S. Vascular biology of the placenta. *In Colloquium Series on Integrated Systems Physiology: From Molecule to Function*. Morgan & Claypool Life Sciences (2010) 2(1):1–98. doi: 10.4199/C00016ED1V01Y201008ISP009
19. Ji L, Brkić J, Liu M, Fu G, Peng C, Wang YL. Placental trophoblast cell differentiation: Physiological regulation and pathological relevance to preeclampsia. *Mol Aspects Med* (2013) 34(5):981–1023. doi: 10.1016/j.mam.2012.12.008
20. Huppertz B. Trophoblast Invasion: Remodelling of Spiral Arteries and Beyond. In: Saito S, editor. *Preeclampsia. Comprehensive Gynecology and Obstetrics*. Springer, Singapore (2018). doi: 10.1007/978-981-10-5891-2_3
21. Frank H-G. Placental Development. In: *Fetal and Neonatal Physiology*. Elsevier: WB Saunders (2017). p. 101–13. doi: 10.1016/B978-0-323-35214-7.00010-X
22. Blaschitz A, Weiss U, Dohr G, Desoye G. Antibody reaction patterns in first trimester placenta: Implications for trophoblast isolation and purity screening. *Placenta* (2000) 21(7):733–41. doi: 10.1053/plac.2000.0559
23. Shorter SC, Starkey PM, Ferry BL, Clover LM, Sargent IL, Redman CWG. Antigenic heterogeneity of human cytotrophoblast and evidence for the transient expression of MHC class I antigens distinct from HLA-G. *Placenta* (1993) 14(5):571–82. doi: 10.1016/S0143-4004(05)80210-3
24. Song H, Ci H, Xu J, Xu Z, Zhang Y, Wang Y, et al. Vasculogenic mimicry and expression of slug and vimentin correlate with metastasis and prognosis in non-small cell lung cancer. *Int J Clin Exp Pathol* (2018) 11(5):2749–58.
25. Lin H, Hong Y, Huang B, Liu X, Zheng J, Qiu S. Vimentin Overexpressions Induced by Cell Hypoxia Promote Vasculogenic Mimicry by Renal Cell Carcinoma Cells. *BioMed Res Int* (2019) 2019:7259691. doi: 10.1155/2019/7259691
26. Hernández de la Cruz ON, López-González JS, García-Vázquez R, Salinas-Vera YM, Muñiz-Lino MA, Aguilar-Cazares D, et al. Regulation Networks Driving Vasculogenic Mimicry in Solid Tumors. *Front Oncol* (2020) 9:1419. doi: 10.3389/fonc.2019.01419
27. Damsky CH, Fitzgerald ML, Fisher SJ. Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, in vivo. *J Clin Invest* (1992) 89(1):210–22. doi: 10.1172/JCI115565

28. Iyengar B, Singh AV. Embryonic vasculogenesis in nodular melanomas and tumour differentiation. *Pathol Oncol Res* (2011) 17(3):569–77. doi: 10.1007/s12253-010-9350-y
29. Liu Y, Li F, Yang YT, Xu XD, Chen JS, Chen TL, et al. IGFBP2 promotes vasculogenic mimicry formation via regulating CD144 and MMP2 expression in glioma. *Oncogene* (2019) 38(11):1815–31. doi: 10.1038/s41388-018-0525-4
30. Zhou Y, Fisher SJ, Janatpour M, Genbacev O, Dejana E, Wheelock M, et al. Human cytotrophoblasts adopt a vascular phenotype as they differentiate: A strategy for successful endovascular invasion? *J Clin Invest* (1997) 99 (9):2139–51. doi: 10.1172/JCI119387
31. Tímár J, Tóvári J, Rásó E, Mészáros L, Bereczky B, Lapis K. Platelet-mimicry of cancer cells: Epiphenomenon with clinical significance. *Oncology* (2005) 69(3):185–201. doi: 10.1159/000088069
32. Damsky CH, Fisher SJ. Trophoblast pseudo-vasculogenesis: Faking it with endothelial adhesion receptors. *Curr Opin Cell Biol* (1998) 10(5):660–6. doi: 10.1016/S0955-0674(98)80043-4
33. Hess AR, Margaryan NV, Seftor EA, Hendrix MJC. Deciphering the signaling events that promote melanoma tumor cell vasculogenic mimicry and their link to embryonic vasculogenesis: Role of the Eph receptors. *Dev Dyn* (2007) 236(12):3283–96. doi: 10.1002/dvdy.21190
34. Wang JY, Sun T, Zhao XL, Zhang SW, Zhang DF, Gu Q, et al. Functional significance of VEGF-a in human ovarian carcinoma: Role in vasculogenic mimicry. *Cancer Biol Ther* (2008) 7(5):758–66. doi: 10.4161/cbt.7.5.5765
35. Burrows TD, King A, Loke YW. Expression of adhesion molecules by endovascular trophoblast and decidual endothelial cells: Implications for vascular invasion during implantation. *Placenta* (1994) 15(1):21–33. doi: 10.1016/S0143-4004(05)80233-4
36. Huppertz B, Kertschanska S, Demir AY, Frank HG, Kaufmann P. Immunohistochemistry of matrix metalloproteinases (MMP), their substrates, and their inhibitors (TIMP) during trophoblast invasion in the human placenta. *Cell Tissue Res* (1997) 291(1):133–48. doi: 10.1007/s004410050987
37. Seftor REB, Seftor EA, Koshikawa N, Meltzer PS, Gardner LMG, Bilban M, et al. Cooperative Interactions of Laminin 5 γ2 Chain, Matrix Metalloproteinase-2, and Membrane Type-1-Matrix/Metalloproteinase Are Required for Mimicry of Embryonic Vasculogenesis by Aggressive Melanoma. *Cancer Res* (2001) 61(17):6322–7.
38. Hurskainen T, Seiki M, Apte SS, Syrjäkallio-Ylitalo M, Sorsa T, Oikarinen A, et al. Production of membrane-type matrix metalloproteinase-1 (MT-MMP-1) in early human placenta. A possible role in placental implantation? *J Histochem Cytochem* (1998) 46(2):221–9. doi: 10.1177/002215549804600211
39. Xu P, Wang Y, Zhu S, Luo S, Piao Y, Zhuang L. Expression of Matrix Metalloproteinase-2, -9, and -14, Tissue Inhibitors of Metalloproteinase-1, and Matrix Proteins in Human Placenta During the First Trimester1. *Biol Reprod* (2000) 62(4):988–94. doi: 10.1095/biolreprod62.4.988
40. Hiden U, Ghaffari-Tabrizi N, Gauster M, Tam-Amersdorfer C, Cetin I, Dieber-Rotheneder M, et al. Membrane-type matrix metalloproteinase 1 regulates trophoblast functions and is reduced in fetal growth restriction. *Am J Pathol* (2013) 182(5):1563–71. doi: 10.1016/j.ajpath.2013.01.011
41. Handschuh K, Guibourdenche J, Tsatsaris V, Guesnon M, Laurendeau I, Evain-Brion D, et al. Human Chorionic Gonadotropin Expression in Human Trophoblasts from Early Placenta: Comparative Study Between Villous and Extravillous Trophoblastic Cells. *Placenta* (2007) 28(2–3):175–84. doi: 10.1016/j.placenta.2006.01.019
42. Selick CE, Horowitz GM, Gratch M, Scott RT, Navot D, Hofmann GE. Immunohistochemical localization of transforming growth factor-beta in human implantation sites. *J Clin Endocrinol Metab* (1994) 78(3):592–6. doi: 10.1210/jcem.78.3.7510303
43. Pietro L, Daher S, Rudge MVC, Calderon IMP, Damasceno DC, Sinzato YK, et al. Vascular endothelial growth factor (VEGF) and VEGF-receptor expression in placenta of hyperglycemic pregnant women. *Placenta* (2010) 31(9):770–80. doi: 10.1016/j.placenta.2010.07.003
44. St-Jacques S, Forte M, Lye SJ, Letarte M. Localization of endoglin, a transforming growth factor-β binding protein, and of CD44 and integrins in placenta during the first trimester of pregnancy. *Biol Reprod* (1994) 51 (3):405–13. doi: 10.1095/biolreprod51.3.405
45. Kovats S, Main EK, Librach C, Stubblebine M, Fisher SJ, Demars R. A class I antigen, HLA-G, expressed in human trophoblasts. *Sci (80-)* (1990) 248 (4952):220–3. doi: 10.1126/science.2326636
46. Moser G, Windsperger K, Pollheimer J, Chuva de Sousa S, Huppertz B. Human trophoblast invasion: new and unexpected routes and functions. *Histochem Cell Biol* (2018) 150:361–70. doi: 10.1007/s00418-018-1699-0
47. Weiss G, Sundl M, Glasner A, Huppertz B, Moser G. The trophoblast plug during early pregnancy: a deeper insight. *Histochem Cell Biol* (2016) 146 (6):749–56. doi: 10.1007/s00418-016-1474-z
48. James JL, Saghian R, Perwick R, Clark AR. Trophoblast plugs: Impact on uteroplacental haemodynamics and spiral artery remodelling. *Hum Reprod* (2018) 33(8):1430–41. doi: 10.1093/humrep/dey225
49. Ander SE, Diamond MS, Coyne CB. Immune responses at the maternal-fetal interface. *Sci Immunol* (2019) 4(31):eaat6114. doi: 10.1126/sciimmunol.aat6114
50. Jauniaux E, Watson AL, Hempstock J, Bao YP, Skepper JN, Burton GJ. Onset of maternal arterial blood flow and placental oxidative stress: A possible factor in human early pregnancy failure. *Am J Pathol* (2000) 157(6):2111–22. doi: 10.1016/S0002-9440(10)64849-3
51. Huppertz B, Weiss G, Moser G. Trophoblast invasion and oxygenation of the placenta: Measurements versus presumptions. *J Reprod Immunol* (2014) 101–102(1):74–9. doi: 10.1016/j.jri.2013.04.003
52. Soares MJ, Iqbal K, Kozai K. Hypoxia and Placental Development. *Birth Defects Res* (2017) 109(17):1309–29. doi: 10.1002/bdr2.1135
53. Sharma S, Godbole G, Modi D. Decidual Control of Trophoblast Invasion. *Am J Reprod Immunol* (2016) 75(3):341–50. doi: 10.1111/aji.12466
54. Weber M, Göhner C, San Martin S, Vattai A, Hutter S, Parraga M, et al. Unique trophoblast stem cell- and pluripotency marker staining patterns depending on gestational age and placenta-associated pregnancy complications. *Cell Adhes Migr* (2016) 10(1–2):56–65. doi: 10.1080/19336918.2016.1142035
55. Mayhew TM. Villous trophoblast of human placenta: A coherent view of its turnover, repair and contributions to villous development and maturation. *Histol Histopathol* (2001) 16(4):1213–24. doi: 10.14670/HH-16.1213
56. Wong FTM, Lin C, Cox BJ. Cellular systems biology identifies dynamic trophoblast populations in early human placentas. *Placenta* (2019) 76:10–8. doi: 10.1016/j.placenta.2018.12.012
57. Chang WL, Liu YW, Dang YL, Jiang XX, Xu H, Huang X, et al. Plac8, a new marker for human interstitial extravillous trophoblast cells, promotes their invasion and migration. *Dev* (2018) 145(2):dev148932. doi: 10.1242/dev.148932
58. Founds SA, Fallert-Junecko B, Reinhart TA, Parks WT. LAIR2-expressing extravillous trophoblasts associate with maternal spiral arterioles undergoing physiologic conversion. *Placenta* (2013) 34(3):248–55. doi: 10.1016/j.placenta.2012.09.017
59. Huppertz B. The anatomy of the normal placenta. *J Clin Pathol* (2008) 61 (12):1296–302. doi: 10.1136/jcp.2008.055277
60. Jansen CHJR, Kastelein AW, Kleinrouweler CE, Van Leeuwen E, De Jong KH, Pajkrt E, et al. Development of placental abnormalities in location and anatomy. *Acta Obstetricia Gynecol Scandinavica* (2020) 99(8):983–93. doi: 10.1111/aogs.13834
61. Rathbun KM, Hildebrand JP. *Placenta Abnormalities*. StatPearls Publishing (2020).
62. Rizov M, Andreeva P, Dimova I. Molecular regulation and role of angiogenesis in reproduction. *Taiwanese J Obstetrics Gynecol* (2017) 56 (2):127–32. doi: 10.1016/j.tjog.2016.06.019
63. Maruo T, Mochizuki M. Immunohistochemical localization of epidermal growth factor receptor and myc oncogene product in human placenta: Implication for trophoblast proliferation and differentiation. *Am J Obstet Gynecol* (1987) 156(3):721–7. doi: 10.1016/0002-9378(87)90086-X
64. Fulop V, Mok SC, Genest DR, Szigetvari I, Cseh I, Berkowitz RS. c-myc, c-erbb-2, c-fms and bcl-2 Oncoproteins: Expression in normal placenta, partial and complete mole, and choriocarcinoma. *J Reprod Med Obstet Gynecol* (1998) 43(2):101–10.
65. Jokhi PP, King A, Loke YW. Reciprocal expression of epidermal growth factor receptor (EGF-R) and c-erbB2 by non-invasive and invasive human trophoblast populations. *Cytokine* (1994) 6(4):433–42. doi: 10.1016/1043-4666(94)90068-X

66. Tarrade A, Lai Kuen R, Malassiné A, Tricottet V, Blain P, Vidaud M, et al. Characterization of human villous and extravillous trophoblasts isolated from first trimester placenta. *Lab Investig* (2001) 81(9):1199–211. doi: 10.1038/labinvest.3780334
67. Wang Y, Cao Y, Sun Y. Expression of c-erbB2 in gestational trophoblastic disease and its clinical significance. *J Huazhong Univ Sci Technol - Med Sci* (2002) 22(2):123–5. doi: 10.1007/bf02857672
68. Rahat B, Hamid A, Najar R, Bagga R, Kaur J. Epigenetic mechanisms regulate placental c-myc and hTERT in normal and pathological pregnancies; c-myc as a novel fetal DNA epigenetic marker for pre-eclampsia. *Mol Hum Reprod* (2014) 20(10):1026–40. doi: 10.1093/MOLEHR/GAU053
69. Xinyuan C, Jiaqin S, Caixia L, Qin Z, Xuesong Y, Shuai L, et al. Progesterone promotes embryo adhesion by upregulating c-Fos/c-Jun transcription factor-mediated poFUT1 expression†. *Biol Reprod* (2019) 101(4):675–85. doi: 10.1093/BIOLRE/IOZ110
70. Peng B, Zhu H, Ma L, Wang YL, Klausen C, Leung PCK. AP-1 transcription factors c-FOS and c-JUN mediate GnRH-induced cadherin-11 expression and trophoblast cell invasion. *Endocrinology* (2015) 156(6):2269–77. doi: 10.1210/en.2014-1871
71. Renaud SJ, Kubota K, Rumi MAK, Soares MJ. The FOS transcription factor family differentially controls trophoblast migration and invasion. *J Biol Chem* (2014) 289(8):5025–39. doi: 10.1074/jbc.M113.523746
72. Goustin AS, Betsholtz C, Pfeifer-Ohlsson S, Persson H, Rydnert J, Bywater M, et al. Coexpression of the sis and myc proto-oncogenes in developing human placenta suggests autocrine control of trophoblast growth. *Cell* (1985) 41(1):301–12. doi: 10.1016/0092-8674(85)90083-2
73. Sarkar S, Kacinski BM, Kohorn EI, Merino MJ, Carter D, Blakemore KJ. Demonstration of myc and ras oncogene expression by hybridization in situ in hydatidiform mole and in the BeWo choriocarcinoma cell line. *Am J Obstet Gynecol* (1986) 154(2):390–3. doi: 10.1016/0002-9378(86)90677-0
74. Liong S, Barker G, Lappas M. Placental ras regulates inflammation associated with maternal obesity. *Mediators Inflamm* (2018) 2018:3645386. doi: 10.1155/2018/3645386
75. Blagoev KB. Cell proliferation in the presence of telomerase. *PloS One* (2009) 4(2):e4622. doi: 10.1371/journal.pone.0004622
76. Kyo S, Takakura M, Tanaka M, Kanaya T, Sagawa T, Kohama T, et al. Expression of telomerase activity in human chorion. *Biochem Biophys Res Commun* (1997) 241(2):498–503. doi: 10.1006/bbrc.1997.7767
77. Chen RJ, Chu CT, Huang SC, Chow SN, Hsieh CY. Telomerase activity in gestational trophoblastic disease and placental tissue from early and late human pregnancies. *Hum Reprod* (2002) 17(2):463–8. doi: 10.1093/humrep/17.2.463
78. Fragkiadaki P, Tsoukalas D, Fragkiadoulaki I, Psycharakis C, Nikitovic D, Spandidos DA, et al. Telomerase activity in pregnancy complications (Review). *Mol Med Rep* (2016) 14(1):16–21. doi: 10.3892/mmr.2016.5231
79. Rama S, Suresh Y, Rao AJ. Regulation of telomerase during human placental differentiation: A role for TGFβ1. *Mol Cell Endocrinol* (2001) 182(2):233–48. doi: 10.1016/S0303-7207(01)00550-0
80. Trybek T, Kowalik A, Góźdź S, Kowalska A. Telomeres and telomerase in oncogenesis (review). *Oncol Lett* (2020) 20(2):1015–27. doi: 10.3892/ol.2020.11659
81. Warrier NM, Agarwal P, Kumar P. Emerging Importance of Survivin in Stem Cells and Cancer: the Development of New Cancer Therapeutics. *Stem Cell Rev Rep* (2020) 16(5):828–52. doi: 10.1007/s12015-020-09995-4
82. Lehner R, Bobak J, Kim NW, Shroyer AL, Shroyer KR. Localization of telomerase hTERT protein and survivin in placenta: Relation to placental development and hydatidiform mole. *Obstet Gynecol* (2001) 97(6):965–70. doi: 10.1016/S0029-7844(01)01131-0
83. Muschol-Steinmetz C, Friemel A, Kreis NN, Reinhard J, Yuan J. and Louwen, F., "Function of Survivin in Trophoblastic Cells of the Placenta. *PloS One* (2013) 8(9):e73337. doi: 10.1371/journal.pone.0073337
84. Burton GJ, Jauniaux E, Murray AJ. Oxygen and placental development; parallels and differences with tumour biology. *Placenta* (2017) 56:14–8. doi: 10.1016/j.placenta.2017.01.130
85. Zhuyan J, Chen M, Zhu T, Bao X, Zhen T, Xing K, et al. Critical steps to tumor metastasis: Alterations of tumor microenvironment and extracellular matrix in the formation of pre-metastatic and metastatic niche. *Cell Biosci* (2020) 10(1):1–9. doi: 10.1186/s13578-020-00453-9
86. Corrado C, Fontana S. Hypoxia and HIF signaling: One axis with divergent effects. *Int J Mol Sci* (2020) 21(6):1–17. doi: 10.3390/ijms21165611
87. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc Natl Acad Sci U S A* (1995) 92(12):5510–4. doi: 10.1073/pnas.92.12.5510
88. Macklin PS, McAuliffe J, Pugh CW. and Yamamoto, A., "Hypoxia and HIF pathway in cancer and the placenta. *Placenta* (2017) 56:8–13. doi: 10.1016/j.placenta.2017.03.010
89. Yang J, Weinberg RA. Epithelial-Mesenchymal Transition: At the Crossroads of Development and Tumor Metastasis. *Dev Cell* (2008) 14 (6):818–29. doi: 10.1016/j.devcel.2008.05.009
90. Davies E, Pollheimer J, Yong HEJ, Kokkinos MI, Kalionis B, Knöfler M, et al. Epithelial-mesenchymal transition during extravillous trophoblast differentiation. *Cell Adhesion Migration* (2016) 10(3):310–21. doi: 10.1080/19336918.2016.1170258
91. Pollheimer J, Fock V, Knöfler M. Review: The ADAM metalloproteinases - Novel regulators of trophoblast invasion? *Placenta* (2014) 35(SUPPL):S57–63. doi: 10.1016/j.placenta.2013.10.012
92. Uekita T, Yamanouchi K, Sato H, Tojo H, Seiki M, Tachi C. Expression and localization of matrix metalloproteinases (MT1-MMP, MMP-2) and tissue inhibitor of metalloproteinase-2 (TIMP-2) during synepitheliochorial placentation of goats (Capra hircus). *Placenta* (2004) 25(10):810–9. doi: 10.1016/j.placenta.2004.03.007
93. Hamutoğlu R, Bulut HE, Kaloğlu C, Önder O, Dağdeviren T, Aydemir MN, et al. The regulation of trophoblast invasion and decidual reaction by matrix metalloproteinase-2, metalloproteinase-7, and metalloproteinase-9 expressions in the rat endometrium. *Reprod Med Biol* (2020) 19(4):385–97. doi: 10.1002/rmb2.12342
94. Plaks V, Rinkenberger J, Dai J, Flannery M, Sund M, Kanasaki K, et al. Matrix metalloproteinase-9 deficiency phenocopies features of preeclampsia and intrauterine growth restriction. *Proc Natl Acad Sci U S A* (2013) 110:11109–14. doi: 10.1073/pnas.1309561110
95. Li M, Liu D, Wang L, Wang W, Wang A, Yao Y. Expression of placenta-specific 8 in human oocytes, embryos, and models of in vitro implantation. *Fertil Steril* (2016) 106(3):781–9.e2. doi: 10.1016/j.fertnstert.2016.05.018
96. Huang CC, Shen MH, Chen SK, Yang SH, Liu CY, Guo JW, et al. Gut butyrate-producing organisms correlate to Placenta Specific 8 protein: Importance to colorectal cancer progression. *J Adv Res* (2020) 22:7–20. doi: 10.1016/j.jare.2019.11.005
97. Hung CS, Wang YC, Guo JW, Yang RN, Lee CL, Shen MH, et al. Expression pattern of placenta specific 8 and keratin 20 in different types of gastrointestinal cancer. *Mol Med Rep* (2020) 21(2):659–66. doi: 10.3892/mmr.2019.10871
98. Zeng X, Liu Q, Yang Y, Jia W, Li S, He D, et al. Placenta-specific protein 8 promotes the proliferation of lung adenocarcinoma PC-9 cells and their tolerance to an epidermal growth factor receptor tyrosine kinase inhibitor by activating the ERK signaling pathway. *Oncol Lett* (2019) 18(5):5621–7. doi: 10.3892/ol.2019.10911
99. Ma X, Liu J, Wang H, Jiang Y, Wan Y, Xia Y, et al. Identification of crucial aberrantly methylated and differentially expressed genes related to cervical cancer using an integrated bioinformatics analysis. *Biosci Rep* (2020) 40(5):BSR20194365. doi: 10.1042/BSR20194365
100. Kaistha BP, Lorenz H, Schmidt H, Sipos B, Pawlak M, Gierke B, et al. PLAC8 localizes to the inner plasma membrane of pancreatic cancer cells and regulates cell growth and disease progression through critical cell-cycle regulatory pathways. *Cancer Res* (2016) 76(1):96–107. doi: 10.1158/0008-5472.CAN-15-0216
101. Tatura M, Schmidt H, Haijat M, Stark M, Rinke A, Diels R, et al. Placenta-Specific 8 Is Overexpressed and Regulates Cell Proliferation in Low-Grade Human Pancreatic Neuroendocrine Tumors. *Neuroendocrinology* (2020) 110 (1–2):23–34. doi: 10.1159/000500541
102. Mao M, Chen Y, Jia Y, Yang J, Wei Q, Li Z, et al. PLCA8 suppresses breast cancer apoptosis by activating the PI3k/AKT/NF-κB pathway. *J Cell Mol Med* (2019) 23(10):6930–41. doi: 10.1111/jcmm.14578

103. Kinsey C, Balakrishnan V, O'Dell MR, Huang JL, Newman L, Whitney-Miller CL, et al. Plac8 links oncogenic mutations to regulation of autophagy and is critical to pancreatic cancer progression. *Cell Rep* (2014) 7(4):1143–55. doi: 10.1016/j.celrep.2014.03.061
104. Demir R, Kayisli UA, Cayli S, Huppertz B. Sequential steps during vasculogenesis and angiogenesis in the very early human placenta. *Placenta* (2006) 27(6–7):535–9. doi: 10.1016/j.placenta.2005.05.011
105. Fernández-Cortés M, Delgado-Bellido D, Oliver FJ. Vasculogenic Mimicry: Become an Endothelial Cell 'But Not So Much'. *Front Oncol* (2019) 9 (AUG):803. doi: 10.3389/fonc.2019.00803
106. Conway EM, Collen D, Carmeliet P. Molecular mechanisms of blood vessel growth. *Cardiovasc Res* (2001) 49(3):507–21. doi: 10.1016/S0008-6363(00)00281-9
107. Demir R, Seval Y, Huppertz B. Vasculogenesis and angiogenesis in the early human placenta. *Acta Histochem* (2007) 109(4):257–65. doi: 10.1016/j.acthis.2007.02.008
108. Nishida N, Yano H, Nishida T, Kamura T, Kojiro M. Angiogenesis in cancer. *Vasc Health Risk Manage* (2006) 2(3):213–9. doi: 10.2147/vhrm.2006.2.3.213
109. Murray MJ, Lessey BA. Embryo implantation and tumor metastasis: Common pathways of invasion and angiogenesis. *Semin Reprod Endocrinol* (1999) 17(3):275–90. doi: 10.1055/s-2007-1016235
110. Brown JM. Vasculogenesis: A crucial player in the resistance of solid tumours to radiotherapy. *Br J Radiol* (2014) 87(1035):20130686. doi: 10.1259/bjr.20130686
111. Testa U, Pelosi E, Castelli G. Endothelial Progenitors in the Tumor Microenvironment. In. *Advances in Experimental Medicine and Biology* Springer (2020) Vol. 1263, pp. 85–115.
112. Li X, Sun X, Carmeliet P. Hallmarks of Endothelial Cell Metabolism in Health and Disease. *Cell Metab* (2019) 30(3):414–33. doi: 10.1016/j.cmet.2019.08.011
113. Zecchin A, Kalucka J, Dubois C, Carmeliet P. How endothelial cells adapt their metabolism to form vessels in tumors. *Front Immunol* (2017) 8:1750 (DEC). doi: 10.3389/fimmu.2017.01750
114. Maquoi E, Van den Brule FA, Castronovo V, Foidart JM. Changes in the distribution pattern of galectin-1 and galectin-3 in human placenta correlates with the differentiation pathways of trophoblasts. *Placenta* (1997) 18(5–6):433–9. doi: 10.1016/S0143-4004(97)80044-6
115. Mourad-Zeidan AA, Melnikova VO, Wang H, Raz A, Bar-Eli M. Expression profiling of galectin-3-depleted melanoma cells reveals its major role in melanoma cell plasticity and vasculogenic mimicry. *Am J Pathol* (2008) 173 (6):1839–52. doi: 10.2353/ajpath.2008.080380
116. Sagrillo-Fagundes L, Bienvenue-Pariseault J, Legembre P, Vaillancourt C. An insight into the role of the death receptor CD95 throughout pregnancy: Guardian, facilitator, or foe. *Birth Defects Res* (2019) 111(4):197–211. doi: 10.1002/bdr2.1470
117. Liu S, Diao L, Huang C, Li Y, Zeng Y, Kwak-Kim JYH. The role of decidual immune cells on human pregnancy. *J Reprod Immunol* (2017) 124:44–53. doi: 10.1016/j.jri.2017.10.045
118. Manaster I, Mandelboim O. The Unique Properties of Uterine NK Cells. *Am J Reprod Immunol* (2010) 63(6):434–44. doi: 10.1111/j.1600-0897.2009.00794.x
119. Nancy P, Erlebacher A. T cell behavior at the maternal-fetal interface. *Int J Dev Biol* (2014) 58(2–4):189–98. doi: 10.1387/ijdb.140054ae
120. Male V, Sharkey A, Masters L, Kennedy PR, Farrell LE, Moffett A. The effect of pregnancy on the uterine NK cell KIR repertoire. *Eur J Immunol* (2011) 41 (10):3017–27. doi: 10.1002/eji.201141445
121. Sojka DK, Yang L, Yokoyama WM. Uterine natural killer cells. *Front Immunol* (2019) 10:960. doi: 10.3389/fimmu.2019.00960
122. Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, et al. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med* (2006) 12(9):1065–74. doi: 10.1038/nm1452
123. Cartwright JE, James-Allan L, Buckley RJ, Wallace AE. The role of decidual NK cells in pregnancies with impaired vascular remodelling. *J Reprod Immunol* (2017) 119:81–4. doi: 10.1016/j.jri.2016.09.002
124. Lash GE, Naruse K, Robson A, Innes BA, Searle RF, Robson SC, et al. Interaction between uterine natural killer cells and extravillous trophoblast cells: effect on cytokine and angiogenic growth factor production. *Hum Reprod* (2011) 26(9):2289–95. doi: 10.1093/humrep/der198
125. Ning F, Liu H, Lash GE. The Role of Decidual Macrophages During Normal and Pathological Pregnancy. *Am J Reprod Immunol* (2016) 75(3):298–309. doi: 10.1111/aji.12477
126. Helige C, Ahammer H, Moser G, Hammer A, Dohr G, Huppertz B, et al. Distribution of decidual natural killer cells and macrophages in the neighbourhood of the trophoblast invasion front: A quantitative evaluation. *Hum Reprod* (2014) 29(1):8–17. doi: 10.1093/humrep/det353
127. Lash GE, Pitman H, Morgan HL, Innes BA, Agwu CN, Bulmer JN. Decidual macrophages: key regulators of vascular remodeling in human pregnancy. *J Leukoc Biol* (2016) 100(2):315–25. doi: 10.1189/jlb.1a0815-351r
128. Morvan MG, Lanier LL. NK cells and cancer: You can teach innate cells new tricks. *Nat Rev Cancer* (2016) 16(1):7–19. doi: 10.1038/nrc.2015.5
129. Vinay DS, Ryan EP, Pawelec G, Talib WH, Stagg J, Elkord E, et al. Immune evasion in cancer: Mechanistic basis and therapeutic strategies. *Semin Cancer Biol* (2015) 35:S185–98. doi: 10.1016/j.semcancer.2015.03.004
130. Wu K, Lin K, Li X, Yuan X, Xu P, Ni P, et al. Redefining Tumor-Associated Macrophage Subpopulations and Functions in the Tumor Microenvironment. *Front Immunol* (2020) 11:1731. doi: 10.3389/fimmu.2020.01731
131. Modiano JF, Bellgrau D. *Fas Ligand Based Immunotherapy: A Potent and Effective Neoadjuvant with Checkpoint Inhibitor Properties, or a Systemically Toxic Promoter of Tumor Growth? - Jaime F Modiano - Discovery Medicine* (2016). Available at: http://www.discoverymedicine.com/Jaime-F-Modiano/2016/02/fas-ligand-based-immunotherapy-a-potent-and-effective-neoadjuvant-with-checkpoint-inhibitor-properties-or-a-systemically-toxic-promoter-of-tumor-growth/ (Accessed Jul. 14, 2020).
132. Houston A, O'Connell J. The Fas signalling pathway and its role in the pathogenesis of cancer. *Curr Opin Pharmacol* (2004) 4(4):321–6. doi: 10.1016/j.coph.2004.03.008
133. Wilczynski JR, Kalinka J, Radwan M. The role of T-regulatory cells in pregnancy and cancer. *Front Biosci* (2008) 13(6):2275–89. doi: 10.2741/2841
134. Zhao JX, Zeng YY, Liu Y. Fetal alloantigen is responsible for the expansion of the CD4+CD25+ regulatory T cell pool during pregnancy. *J Reprod Immunol* (2007) 75(2):71–81. doi: 10.1016/j.jri.2007.06.052
135. Loustau M, Anna F, Dréan R, Lecomte M, Langlade-Demoyen P, Caumartin J. HLA-G Neo-Expression on Tumors. *Front Immunol* (2020) 11:1685. doi: 10.3389/fimmu.2020.01685
136. Lin A, Yan WH. Intercellular transfer of HLA-G: its potential in cancer immunology. *Clin Trans Immunol* (2019) 8(9):e1077. doi: 10.1002/cti2.1077
137. Gimenes F, Teixeira JJV, de Abreu ALP, Souza RP, Pereira MW, da Silva VRS, et al. Human leukocyte antigen (HLA)-G and cervical cancer immunoediting: A candidate molecule for therapeutic intervention and prognostic biomarker? *Biochim Biophys Acta - Rev Cancer* (2014) 1846 (2):576–89. doi: 10.1016/j.bbcan.2014.10.004
138. Steven A, Seliger B. The Role of Immune Escape and Immune Cell Infiltration in Breast Cancer. *Breast Care* (2018) 13(1):16–21. doi: 10.1159/000486585
139. Farjadian S, Tabebordbar M, Mokhtari M, Safaei A, Malekzadeh M, Gaderi A. HLA-G expression in tumor tissues and soluble HLA-G plasma levels in patients with gastrointestinal cancer. *Asian Pacific J Cancer Prev* (2018) 19(10):2731–5. doi: 10.22034/APJCP.2018.19.10.2731
140. Mijaljica D, Prescott M, Devenish RJ. Microautophagy in mammalian cells: Revisiting a 40-year-old conundrum. *Autophagy* (2011) 7(7):673–82. doi: 10.4161/auto.7.7.14733
141. Wu H, Chen S, Ammar AB, Xu J, Wu Q, Pan K, et al. Crosstalk Between Macroautophagy and Chaperone-Mediated Autophagy: Implications for the Treatment of Neurological Diseases. *Mol Neurobiol* (2015) 52(3):1284–96. doi: 10.1007/s12035-014-8933-0
142. Mehrpour M, Esclatine A, Beau I, Codogno P. Overview of macroautophagy regulation in mammalian cells. *Cell Res* (2010) 20(7):748–62. doi: 10.1038/cr.2010.82
143. Parzych KR, Klionsky DJ. An overview of autophagy: Morphology, mechanism, and regulation. *Antioxid Redox Signaling* (2014) 20(3):460–73. doi: 10.1089/ars.2013.5371
144. Eskelinen EL, Saftig P. Autophagy: A lysosomal degradation pathway with a central role in health and disease. *Biochim Biophys Acta - Mol Cell Res* (2009) 1793(4):664–73. doi: 10.1016/j.bbamcr.2008.07.014

145. Kaur J, Debnath J. Autophagy at the crossroads of catabolism and anabolism. *Nat Rev Mol Cell Biol* (2015) 16(8):461–72. doi: 10.1038/nrm4024
146. Ravikumar B, Sarkar S, Davies JE, Futter M, Garcia-Arencibia M, Green-Thompson ZW, et al. Regulation of Mammalian Autophagy in Physiology and Pathophysiology. *Physiol Rev* (2010) 90(4):1383–435. doi: 10.1152/physrev.00030.2009
147. Reggiori F, Ungermann C. Autophagosome Maturation and Fusion. *J Mol Biol* (2017) 429(4):486–96. doi: 10.1016/j.jmb.2017.01.002
148. Mizushima N, Komatsu M. Autophagy: Renovation of cells and tissues. *Cell* (2011) 147(4):728–41. doi: 10.1016/j.cell.2011.10.026
149. Itakura E, Mizushima N. Characterization of autophagosome formation site by a hierarchical analysis of mammalian Atg proteins. *Autophagy* (2010) 6 (6):764–76. doi: 10.4161/auto.6.6.12709
150. Reggiori F, Tucker KA, Stromhaug PE. The Atg1-Atg13 complex regulates Atg9 and Atg23 retrieval transport from the pre-autophagosomal structure. *Dev Cell* (2004) 6(1):79–90. doi: 10.1016/S1534-5807(03)00402-7
151. Yamamoto H, Kakuta S, Watanabe TM, Kitamura A, Sekito T, Kondo-Kakuta C, et al. Atg9 vesicles are an important membrane source during early steps of autophagosome formation. *J Cell Biol* (2012) 198(2):219–33. doi: 10.1083/jcb.201202061
152. Ohsumi Y, Mizushima N. Two ubiquitin-like conjugation systems essential for autophagy. *Semin Cell Dev Biol* (2004) 15(2):231–6. doi: 10.1016/j.semcdb.2003.12.004
153. Hanada T, Noda NN, Satomi Y, Ichimura Y, Fujioka Y, Takao T, et al. The Atg12-Atg5 Conjugate Has a Novel E3-like Activity for Protein Lipidation in Autophagy. *J Biol Chem* (2007) 282(52):37298–302. doi: 10.1074/jbc.C700195200
154. Johansen T, Lamark T. Selective autophagy mediated by autophagic adapter proteins. *Autophagy* (2011) 7(3):279–96. doi: 10.4161/auto.7.3.14487
155. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, et al. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy*[S]. *J Biol Chem* (2007) 282 (33):24131–45. doi: 10.1074/jbc.M702824200
156. Takahashi Y, He H, Tang Z, Hattori T, Liu Y, Young MM, et al. An autophagy assay reveals the ESCRT-III component CHMP2A as a regulator of phagophore closure. *Nat Commun* (2018) 9(1):1–13. doi: 10.1038/s41467-018-05254-w
157. Yu S, Melia TJ. The coordination of membrane fission and fusion at the end of autophagosome maturation. *Curr Opin Cell Biol* (2017) 47:92–8. doi: 10.1016/j.ceb.2017.03.010
158. Zhao YG, Zhang H. Autophagosome maturation: An epic journey from the ER to lysosomes. *J Cell Biol* (2019) 218(3):757–70. doi: 10.1083/jcb.201810099
159. Korolchuk VI, Rubinsztein DC. Regulation of autophagy by lysosomal positioning. *Autophagy* (2011) 7(8):927–8. doi: 10.4161/auto.7.8.15862
160. Nakamura S, Yoshimori T. New insights into autophagosome-lysosome fusion. *J Cell Sci* (2017) 130(7):1209–16. doi: 10.1242/jcs.196352
161. Yu L, Chen Y, Tooze SA. Autophagy pathway: Cellular and molecular mechanisms. *Autophagy* (2018) 14(2):207–15. doi: 10.1080/15548627.2017.1378838
162. Rong Y, McPhee C, Denga S, Huanga L, Chen L, Liu M, et al. Spinster is required for autophagic lysosome reformation and mTOR reactivation following starvation. *Proc Natl Acad Sci U S A* (2011) 108(19):7826–31. doi: 10.1073/pnas.1013800108
163. Yu L, McPhee CK, Zheng L, Mardones GA, Rong Y, Peng J, et al. Termination of autophagy and reformation of lysosomes regulated by mTOR. *Nature* (2010) 465(7300):942–6. doi: 10.1038/nature09076
164. Kawabata T, Yoshimori T. Autophagosome biogenesis and human health. *Cell Discovery* (2020) 6(1):1–14. doi: 10.1038/s41421-020-0166-y
165. Fujikake N, Shin M, Shimizu S. Association between autophagy and neurodegenerative diseases. *Front Neurosci* (2018) 12(MAY):255. doi: 10.3389/fnins.2018.00255
166. Netea-Maier RT, Plantinga TS, van de Veerdonk FL, Smit JW, Netea MG. Modulation of inflammation by autophagy: Consequences for human disease. *Autophagy* (2016) 12(2):245–60. doi: 10.1080/15548627.2015.1071759
167. Rocchi A, He C. Emerging roles of autophagy in metabolism and metabolic disorders. *Front Biol* (2015) 10(2):154–64. doi: 10.1007/s11515-015-1354-2
168. Ryter SW, Koo JK, Choi AMK. Molecular regulation of autophagy and its implications for metabolic diseases. *Curr Opin Clin Nutr Metab Care* (2014) 17(4):329–37. doi: 10.1097/MCO.0000000000000068
169. Yang Y, Klionsky DJ. Autophagy and disease: unanswered questions. *Cell Death Differentiation* (2020) 27(3):858–71. doi: 10.1038/s41418-019-0480-9
170. Nakashima A, Shima T, Aoki A, Kawaguchi M, Yasuda I, Tsuda S, et al. Molecular and immunological developments in placentas. *Hum Immunol* (2021). doi: 10.1016/j.humimm.2021.01.012
171. Lu H, Yang HL, Zhou WJ, Lai ZZ, Qiu XM, Fu Q, et al. Rapamycin prevents spontaneous abortion by triggering decidual stromal cell autophagy-mediated NK cell residence. *Autophagy* (2020) 1:1–17. doi: 10.1080/15548627.2020.1833515
172. Tan HX, Yang SL, Li MQ, Wang HY. Autophagy suppression of trophoblast cells induces pregnancy loss by activating decidual NK cytotoxicity and inhibiting trophoblast invasion. *Cell Commun Signal* (2020) 18(1):1–16. doi: 10.1186/s12964-020-00579-w
173. Noman MZ, Janji B, Berchem G, Mami-Chouaib F, Chouaib S. Hypoxia-induced autophagy: A new player in cancer immunotherapy? *Autophagy* (2012) 8(4):704–6. doi: 10.4161/auto.19572
174. Janji B, Berchem G, Chouaib S. Targeting autophagy in the tumor microenvironment: New challenges and opportunities for regulating tumor immunity. *Front Immunol* (2018) 9:887(APR). doi: 10.3389/fimmu.2018.00887
175. Tittarelli A, Janji B, Van Moer K, Noman MZ, Chouaib S. The selective degradation of synaptic connexin 43 protein by hypoxia-induced autophagy impairs natural killer cell-mediated tumor cell killing. *J Biol Chem* (2015) 290 (39):23670–9. doi: 10.1074/jbc.M115.651547
176. Gerada C, Ryan KM. Autophagy, the innate immune response and cancer. *Mol Oncol* (2020) 14(9):1913–29. doi: 10.1002/1878-0261.12774
177. Jiang GM, Tan Y, Wang H, Peng L, Chen HT, Meng XJ, et al. The relationship between autophagy and the immune system and its applications for tumor immunotherapy. *Mol Cancer* (2019) 18(1):1–22. doi: 10.1186/s12943-019-0944-z
178. Baginska J, Viry E, Berchem G, Poli A, Noman MZ, Van Moer K, et al. Granzyme B degradation by autophagy decreases tumor cell susceptibility to natural killer-mediated lysis under hypoxia. *Proc Natl Acad Sci U S A* (2013) 110(43):17450–5. doi: 10.1073/pnas.1304790110
179. Yao C, Ni Z, Gong C, Zhu X, Wang L, Xu Z, et al. Rocaglamide enhances NK cell-mediated killing of non-small cell lung cancer cells by inhibiting autophagy. *Autophagy* (2018) 14(10):1831–44. doi: 10.1080/15548627.2018.1489946
180. Zhao H, Jia H, Han Q, Zhang J. Homeobox containing 1 inhibits liver cancer progression by promoting autophagy as well as inhibiting stemness and immune escape. *Oncol Rep* (2018) 40(3):1657–65. doi: 10.3892/or.2018.6551
181. Zaarour RF, Azakir B, Hajam EY, Nawafleh H, Zeinelabdin NA, Engelsen AST, et al. Role of hypoxia-mediated autophagy in tumor cell death and survival. *Cancers* (2021) 13(3):1–20. doi: 10.3390/cancers13030533
182. Chifenti B, Locci MT, Lazzeri G, Guagnozzi M, Dinucci D, Chiellini F, et al. Autophagy-related protein LC3 and Beclin-1 in the first trimester of pregnancy. *Clin Exp Reprod Med* (2013) 40(1):33–7. doi: 10.5653/cerm.2013.40.1.33
183. Signorelli P, Avagliano L, Virgili E, Gagliostro V, Doi P, Braidotti P, et al. Autophagy in term normal human placentas. *Placenta* (2011) 32(6):482–5. doi: 10.1016/j.placenta.2011.03.005
184. Chen GQ, Zhang H, Qi H, Yao ZW, Gao L, Qiu CL. [Effects and mechanisms of autophagy of trophoblast cells in severe preeclampsia]. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* (2012) 28(3):294–6.
185. Hung TH, Chen SF, Lo LM, Li MJ, Yeh YL, Hsieh TT. an., "Increased autophagy in placentas of intrauterine growth-restricted pregnancies. *PloS One* (2012) 7(7):e40957. doi: 10.1371/journal.pone.0040957
186. Choi J-H, Lee H-J, Yang T-H, Kim GJ. Effects of hypoxia inducible factors-1α on autophagy and invasion of trophoblasts. *Clin Exp Reprod Med* (2012) 39(2):73–80. doi: 10.5653/cerm.2012.39.2.73
187. Chen B, Longtine MS, Nelson DM. Hypoxia induces autophagy in primary human trophoblasts. *Endocrinology* (2012) 153(10):4946–54. doi: 10.1210/en.2012-1472

188. Chang YL, Wang TH, Chang SD, Chao AS, Hsieh PCC. and Wang, C. N., "Increased autophagy in the placental territory of selective intrauterine growth-restricted monochorionic twins. *Prenat Diagn* (2013) 33(2):187–90. doi: 10.1002/pd.4040
189. Nakashima A, Yamanaka-Tatematsu M, Fujita N, Koizumi K, Shima T, Yoshida T, et al. Impaired autophagy by soluble endoglin, under physiological hypoxia in early pregnant period, is involved in poor placentation in preeclampsia. *Autophagy* (2013) 9(3):303–16. doi: 10.4161/auto.22927
190. Avagliano L, Virgili E, Garò C, Quadrelli F, Doi P, Samaja M, et al. Autophagy and human parturition: Evaluation of LC3 expression in placenta from spontaneous or medically induced onset of labor. *BioMed Res Int* (2013) 2013:1–9. doi: 10.1155/2013/689768
191. Avagliano L, Danti L, Doi P, Felis S, Guala M, Locatelli A, et al. Autophagy in placentas from acidotic newborns: An immunohistochemical study of LC3 expression. *Placenta* (2013) 34(11):1091–4. doi: 10.1016/j.placenta.2013.09.004
192. Yamanaka-Tatematsu M, Nakashima A, Fujita N, Shima T, Yoshimori T, Saito S. Autophagy induced by HIF1α overexpression supports trophoblast invasion by supplying cellular energy. *PloS One* (2013) 8(10):e76605. doi: 10.1371/journal.pone.0076605
193. Kalkat M, Garcia J, Ebrahimi J, Melland-Smith M, Todros T, Post M, et al. Placental autophagy regulation by the BOK-MCL1 rheostat. *Autophagy* (2013) 9(12):2140–53. doi: 10.4161/auto.26452
194. Hung T-H, Hsieh T-T, Chen S-F, Li M-J, Yeh Y-L. Autophagy in the Human Placenta throughout Gestation. *PloS One* (2013) 8(12):e83475. doi: 10.1371/journal.pone.0083475
195. Cha HH, Hwang JR, Kim HY, Choi SJ, Oh SY, Roh CR. Autophagy induced by tumor necrosis factor mediates intrinsic apoptosis in trophoblastic cells. *Reprod Sci* (2014) 21(5):612–22. doi: 10.1177/1933719113508816
196. Akaishi R, Yamada T, Nakabayashi K, Nishihara H, Furuta I, Kojima T, et al. Autophagy in the placenta of women with hypertensive disorders in pregnancy. *Placenta* (2014) 35(12):974–80. doi: 10.1016/j.placenta.2014.10.009
197. Gaoa L, Qi HB, Kc K, Zhang XM, Zhang H, Baker PN. Excessive autophagy induces the failure of trophoblast invasion and vasculature: Possible relevance to the pathogenesis of preeclampsia. *J Hypertens* (2015) 33(1):106–17. doi: 10.1097/HJH.0000000000000366
198. Avagliano L, Terraneo L, Virgili E, Martinelli C, Doi P, Samaja M, et al. Autophagy in normal and abnormal early human pregnancies. *Reprod Sci* (2015) 22(7):838–44. doi: 10.1177/1933719114565036
199. Melland-Smith M, Ermini L, Chauvin S, Craig-Barnes H, Tagliaferro A, Todros T, et al. Disruption of sphingolipid metabolism augments ceramide-induced autophagy in preeclampsia. *Autophagy* (2015) 11(4):653–69. doi: 10.1080/15548627.2015.1034414
200. Brickle A, Tran HT, Lim R, Liong S, Lappas M. Autophagy, which is decreased in labouring fetal membranes, regulates IL-1β production via the inflammasome. *Placenta* (2015) 36(12):1393–404. doi: 10.1016/j.placenta.2015.10.015
201. He B, Zhang N, Zhao R. Dexamethasone Downregulates SLC7A5 Expression and Promotes Cell Cycle Arrest, Autophagy and Apoptosis in BeWo Cells. *J Cell Physiol* (2016) 231(1):233–42. doi: 10.1002/jcp.25076
202. Nakabayashi Y, Nakashima A, Yoshino O, Shima T, Shiozaki A, Adachi T, et al. Impairment of the accumulation of decidual T cells, NK cells, and monocytes, and the poor vascular remodeling of spiral arteries, were observed in oocyte donation cases, regardless of the presence or absence of preeclampsia. *J Reprod Immunol* (2016) 114:65–74. doi: 10.1016/j.jri.2015.07.005
203. Wang Y, Chen B, Longtine MS, Nelson DM. Punicalagin promotes autophagy to protect primary human syncytiotrophoblasts from apoptosis. *Reproduction* (2016) 151(2):97–104. doi: 10.1530/REP-15-0287
204. Muralimanoharan S, Gao X, Weintraub S, Myatt L, Maloyan A. Sexual dimorphism in activation of placental autophagy in obese women with evidence for fetal programming from a placenta-specific mouse model. *Autophagy* (2016) 12(5):752–69. doi: 10.1080/15548627.2016.1156822
205. He MY, Wang G, Han SS, Jin Y, Li H, Wu X, et al. Nrf2 signalling and autophagy are involved in diabetes mellitus-induced defects in the development of mouse placenta. *Open Biol* (2016) 6(7):160064. doi: 10.1098/rsob.160064
206. Zhang Y, Hu X, Gao G, Wang Y, Chen P, Ye Y. Autophagy protects against oxidized low density lipoprotein-mediated inflammation associated with preeclampsia. *Placenta* (2016) 48:136–43. doi: 10.1016/j.placenta.2016.09.015
207. Cao B, Macones C, Mysorekar IU. ATG16L1 governs placental infection risk and preterm birth in mice and women. *JCI Insight* (2016) 1(21):e86654. doi: 10.1172/jci.insight.86654
208. Xu Y, Huang X, Xie J, Chen Y, Fu J, Wang L. Let-7i-Induced Atg4B Suppression Is Essential for Autophagy of Placental Trophoblast in Preeclampsia. *J Cell Physiol* (2017) 232(9):2581–9. doi: 10.1002/jcp.25661
209. Avagliano L, Massa V, Zullino S, Doi P, Marconi AM, Ferrazzi E, et al. Inflammation modulates LC3 expression in human preterm delivery. *J Matern Neonatal Med* (2017) 30(6):698–704. doi: 10.1080/14767058.2016.1183630
210. Prokesch A, Blaschitz A, Bauer T, Moser G, Hiden U, Zadora J, et al. Placental DAPK1 and autophagy marker LC3B-II are dysregulated by TNF-α in a gestational age-dependent manner. *Histochem Cell Biol* (2017) 147(6):695–705. doi: 10.1007/s00418-016-1537-1
211. Avagliano L, Massa V, Terraneo L, Samaja M, Doi P, Bulfamante GP, et al. Gestational diabetes affects fetal autophagy. *Placenta* (2017) 55:90–3. doi: 10.1016/j.placenta.2017.05.002
212. Zhang QX, Na Q, Song W. Altered expression of mTOR and autophagy in term normal human placentas. *Rom J Morphol Embryol* (2017) 2012(1):61–6.
213. Ji L, Chen Z, Xu Y, Xiong G, Liu R, Wu C, et al. Systematic Characterization of Autophagy in Gestational Diabetes Mellitus. *Endocrinology* (2017) 158(8):2522–32. doi: 10.1210/en.2016-1922
214. Holland OJ, Hickey AJR, Alvsaker A, Moran S, Hedges C, Chamley LW, et al. Changes in mitochondrial respiration in the human placenta over gestation. *Placenta* (2017) 57:102–12. doi: 10.1016/j.placenta.2017.06.011
215. Hutabarat M, Wibowo N, Huppertz B. The trophoblast survival capacity in preeclampsia. *PloS One* (2017) 12(11):e0186909. doi: 10.1371/journal.pone.0186909
216. Dimasuay KG, Gong L, Rosario F, McBryde E, Spelman T, Glazier J, et al. Impaired placental autophagy in placental malaria. *PloS One* (2017) 12(11):e0187291. doi: 10.1371/journal.pone.0187291
217. Pan T, He G, Chen M, Bao C, Chen Y, Liu G, et al. Abnormal CYP11A1 gene expression induces excessive autophagy, contributing to the pathogenesis of preeclampsia. *Oncotarget* (2017) 8(52):89824–36. doi: 10.18632/oncotarget.21158
218. Hung TH, Hsieh TT, Wu CP, Li MJ, Yeh YL, Chen SF. Mammalian target of rapamycin signaling is a mechanistic link between increased endoplasmic reticulum stress and autophagy in the placentas of pregnancies complicated by growth restriction. *Placenta* (2017) 60:9–20. doi: 10.1016/j.placenta.2017.10.001
219. Akcora Yildiz D, Irtegun Kandemir S, Agacayak E, Deveci E. Evaluation of protein levels of autophagy markers (Beclin 1 and SQSTM1/p62) and phosphorylation of cyclin E in the placenta of women with preeclampsia. *Cell Mol Biol* (2017) 63(12):51. doi: 10.14715/cmb/2017.63.12.12
220. Lee H-M, Choi K-C. Cigarette smoke extract and isoprene resulted in the induction of apoptosis and autophagy in human placenta choriocarcinoma JEG-3 cells. *Environ Toxicol* (2018) 33(2):178–90. doi: 10.1002/tox.22506
221. Gauster M, Maninger S, Siwetz M, Deutsch A, El-Heliebi A, Kolb-Lenz D, et al. Downregulation of p53 drives autophagy during human trophoblast differentiation. *Cell Mol Life Sci* (2018) 75(10):1839–55. doi: 10.1007/s00018-017-2695-6
222. Hong Y-J, Ahn H-J, Shin J, Lee JH, Kim J-H, Park H-W, et al. Unsaturated fatty acids protect trophoblast cells from saturated fatty acid-induced autophagy defects. *J Reprod Immunol* (2018) 125:56–63. doi: 10.1016/j.jri.2017.12.001
223. Hawkins SJ, Crompton LA, Sood A, Saunders M, Boyle NT, Buckley A, et al. Nanoparticle-induced neuronal toxicity across placental barriers is mediated by autophagy and dependent on astrocytes. *Nat Nanotechnol* (2018) 13(5):427–33. doi: 10.1038/s41565-018-0085-3
224. Ozsoy AZ, Cayli S, Sahin C, Ocakli S, Sanci TO, Ilhan DB. Altered expression of p97/Valosin containing protein and impaired autophagy in preeclamptic human placenta. *Placenta* (2018) 67:45–53. doi: 10.1016/j.placenta.2018.05.013

225. Pan YJ, He L, Zhou SJ, Zhang LJ, Zhang AH, Zhao YY. Expression of urotensin II is associated with placental autophagy in patients with severe preeclampsia. *J Hum Hypertens* (2018) 32(11):759–69. doi: 10.1038/s41371-018-0083-9
226. Sagrillo-Fagundes L, Assunção Salustiano EM, Ruano R, Markus RP, Vaillancourt C. Melatonin modulates autophagy and inflammation protecting human placental trophoblast from hypoxia/reoxygenation. *J Pineal Res* (2018) 65(4):e12520. doi: 10.1111/jpi.12520
227. Hu J, Liu L, Gong Y, Zhang L, Gan X, Luo X, et al. Linc02527 promoted autophagy in Intrahepatic cholestasis of pregnancy. *Cell Death Dis* (2018) 9 (10):1–14. doi: 10.1038/s41419-018-1013-z
228. Zhang Y, Xu B, Yao M, Dong T, Mao Z, Hang B, et al. Titanium dioxide nanoparticles induce proteostasis disruption and autophagy in human trophoblast cells. *Chem Biol Interact* (2018) 296:124–33. doi: 10.1016/j.cbi.2018.09.015
229. Cai H, Chen L, Zhang M, Xiang W, Su P. Low expression of MFN2 is associated with early unexplained miscarriage by regulating autophagy of trophoblast cells. *Placenta* (2018) 70:34–40. doi: 10.1016/j.placenta.2018.08.005
230. Mao Z, Yao M, Li Y, Fu Z, Li S, Zhang L, et al. MiR-96-5p and miR-101-3p as potential intervention targets to rescue TiO2 NP-induced autophagy and migration impairment of human trophoblastic cells. *Biomater Sci* (2018) 6 (12):3273–83. doi: 10.1039/c8bm00856f
231. Zhang L, Deng X, Shi X, Dong X. Silencing H19 regulated proliferation, invasion, and autophagy in the placenta by targeting miR-18a-5p. *J Cell Biochem* (2019) 120(6):9006–15. doi: 10.1002/jcb.28172
232. Sagrillo-Fagundes L, Bienvenue-Pariseault J, Vaillancourt Id C. Melatonin: The smart molecule that differentially modulates autophagy in tumor and normal placental cells. *PloS One* (2019) 14(1):e0202458. doi: 10.1371/journal.pone.0202458
233. Nakashima A, Higashisaka K, Kusabiraki T, Aoki A, Ushijima A, Ono Y, et al. Autophagy is a new protective mechanism against the cytotoxicity of platinum nanoparticles in human trophoblasts. *Sci Rep* (2019) 9(1):1–13. doi: 10.1038/s41598-019-41927-2
234. Wang Y, Liu LL, Tian Y, Chen Y, Zha WH, Li Y, et al. Upregulation of DAPK2 ameliorates oxidative damage and apoptosis of placental cells in hypertensive disorder complicating pregnancy by suppressing human placental microvascular endothelial cell autophagy through the mTOR signaling pathway. *Int J Biol Macromol* (2019) 121:488–97. doi: 10.1016/j.ijbiomac.2018.09.111
235. Chen Y, Wang L, Bao J, Sha X, Cui L, Huang Q, et al. Persistent hypoxia induced autophagy leading to invasiveness of trophoblasts in placenta accreta. *J Matern Neonatal Med* (2019) 34(8):1291–303. doi: 10.1080/14767058.2019.1635582
236. Nakashima A, Cheng SB, Kusabiraki T, Motomura K, Aoki A, Ushijima A, et al. Endoplasmic reticulum stress disrupts lysosomal homeostasis and induces blockade of autophagic flux in human trophoblasts. *Sci Rep* (2019) 9 (1):1–12. doi: 10.1038/s41598-019-47607-5
237. Zhu HL, Xu XF, Shi XT, Feng YJ, Xiong YW, Nan Y, et al. Activation of autophagy inhibits cadmium-triggered apoptosis in human placental trophoblasts and mouse placenta. *Environ Pollut* (2019) 254(Pt A). doi: 10.1016/j.envpol.2019.112991
238. Bastida-Ruiz D, Yart L, Wuillemin C, Ribaux P, Morris N, Epiney M, et al. The fine-tuning of endoplasmic reticulum stress response and autophagy activation during trophoblast syncytialization. *Cell Death Dis* (2019) 10 (9):651. doi: 10.1038/s41419-019-1905-6
239. Lima FA, Barateiro A, Dombrowski JG, de Souza RM, de Sousa Costa D, Murillo O, et al. Plasmodium falciparum infection dysregulates placental autophagy. *PloS One* (2019) 14(12):e0226117. doi: 10.1371/journal.pone.0226117
240. Wang Y, Ji L, Peng Z, Lai R, Zhang X, Xu Y, et al. Silencing DAPK3 blocks the autophagosome-lysosome fusion by mediating SNAP29 in trophoblast cells under high glucose treatment. *Mol Cell Endocrinol* (2020) 502. doi: 10.1016/j.mce.2019.110674
241. Zhang H, He Y, Wang JX, Chen MH, Xu JJ, Jiang MH, et al. miR-30-5p-mediated ferroptosis of trophoblasts is implicated in the pathogenesis of preeclampsia. *Redox Biol* (2020) 29:101402. doi: 10.1016/j.redox.2019.101402
242. Wang P, Huang CX, Gao JJ, Shi Y, Li H, Yan H, et al. Resveratrol induces SIRT1-Dependent autophagy to prevent H2O2-Induced oxidative stress and apoptosis in HTR8/SVneo cells. *Placenta* (2020) 91:11–8. doi: 10.1016/j.placenta.2020.01.002
243. Yang D, Ding J, Wang Y, Yuan M, Xian S, Zhang L, et al. YY1-PVT1 affects trophoblast invasion and adhesion by regulating mTOR pathway-mediated autophagy. *J Cell Physiol* (2020) 235(10):6637–46. doi: 10.1002/jcp.29560
244. Han Q, Zhang W, Lu C, Wu J, An S, Zhang S. Repression of Kisspeptin1 weakens hydrogen peroxide-caused injury in HTR8 cells via adjusting PI3K/AKT/mTOR pathway. *J Biochem Mol Toxicol* (2020) 34(5):e22461. doi: 10.1002/jbt.22461
245. Hung TH, Huang SY, Chen SF, Wu CP, Hsieh TT. an., "Decreased placental apoptosis and autophagy in pregnancies complicated by gestational diabetes with large-for-gestational age fetuses. *Placenta* (2020) 90:27–36. doi: 10.1016/j.placenta.2019.12.003
246. Mao Q, Chu S, Shapiro S, Yao H, De Paepe ME. Discordant placental oxygenation and autophagy in twin anemia-polycythemia sequence (TAPS). *Placenta* (2020) 90:9–17. doi: 10.1016/j.placenta.2019.11.003
247. Zaugg J, Melhem H, Huang X, Wegner M, Baumann M, Surbek D, et al. Gestational diabetes mellitus affects placental iron homeostasis: Mechanism and clinical implications. *FASEB J* (2020) 34(6):7311–29. doi: 10.1096/fj.201903054R
248. Yang P, Li Z, Tye KD, Chen Y, Lu T, He Z, et al. Effects of an orally supplemented probiotic on the autophagy protein LC3 and Beclin1 in placentas undergoing spontaneous delivery during normal pregnancy. *BMC Pregnancy Childbirth* (2020) 20(1):1–8. doi: 10.1186/s12884-020-02905-z
249. Cohen M, Guo E, Pucchio A, de Vrijer B, Shepherd TG, Eastabrook G. Maternal obesity reduces placental autophagy marker expression in uncomplicated pregnancies. *J Obstet Gynaecol Res* (2020) 46(8):1282–91. doi: 10.1111/jog.14315
250. Dong X, Zhang Y, Chen X, Xue M. Long noncoding RNA LINC00511 regulates the proliferation, apoptosis, invasion and autophagy of trophoblast cells to mediate pre-eclampsia progression through modulating the miR-31-5p/homeobox protein A7 axis. *J Obstet Gynaecol Res* (2020) 46(8):1298–309. doi: 10.1111/jog.14344
251. Du J, Ji Q, Dong L, Meng Y, Xin G. HDAC4 Knockdown Induces Preeclampsia Cell Autophagy and Apoptosis by miR-29b. *Reprod Sci* (2020) 28(2):334–42. doi: 10.1007/s43032-020-00286-4
252. Ji Y, Zhang W, Yang J, Li C. MiR-193b inhibits autophagy and apoptosis by targeting IGFBP5 in high glucose-induced trophoblasts. *Placenta* (2020) 101:185–93. doi: 10.1016/j.placenta.2020.09.015
253. Li YX, Long DL, Liu J, Qiu D, Wang J, Cheng X, et al. Gestational diabetes mellitus in women increased the risk of neonatal infection via inflammation and autophagy in the placenta. *Med (Baltimore)* (2020) 99(40):e22152. doi: 10.1097/MD.0000000000022152
254. Pan Y, Yan L, Chen Q, Wei C, Dai Y, Tong X, et al. Dysfunction of Shh signaling activates autophagy to inhibit trophoblast motility in recurrent miscarriage. *Exp Mol Med* (2021) 53(1):52–66. doi: 10.1038/s12276-020-00530-6
255. de la Torre P, Fernández-de la Torre M, Flores AI. Premature senescence of placental decidua cells as a possible cause of miscarriage produced by mycophenolic acid. *J Biomed Sci* (2021) 28(1):1–14. doi: 10.1186/s12929-020-00704-4
256. Dai Y, Li TH, He X, Yan SB, Gao Y, Chen Y. The Effect and Mechanism of Asymmetric Dimethylarginine Regulating Trophoblastic Autophagy on Fetal Growth Restriction. *Reprod Sci* (2021) 1–11. doi: 10.1007/s43032-020-00442-w
257. Vangrieken P, Al-Nasiry S, Bast A, Leermakers PA, Tulen CBM, Janssen GMJ, et al. Hypoxia-induced mitochondrial abnormalities in cells of the placenta. *PloS One* (2021) 16(1):e0245155. doi: 10.1371/journal.pone.0245155
258. Chen Z, Li C, Yuan A, Gu T, Zhang F, Fan X, et al. α-Solanine Causes Cellular Dysfunction of Human Trophoblast Cells via Apoptosis and Autophagy. *Toxins (Basel)* (2021) 13(1):67. doi: 10.3390/toxins13010067
259. Vangrieken P, Al-Nasiry S, Bast A, Leermakers PA, Tulen CBM, Schiffers PMH, et al. Placental Mitochondrial Abnormalities in Preeclampsia. *Reprod Sci* (2021) 1–14. doi: 10.1007/s43032-021-00464-y

260. Feng X, Wei Z, Tao X, Du Y, Wu J, Yu Y, et al. PLAC8 promotes the autophagic activity and improves the growth priority of human trophoblast cells. *FASEB J* (2021) 35(3):e21351. doi: 10.1096/fj.202002075rr
261. Curtis S, Jones CJP, Garrod A, Hulme CH, Heazell AEP. Identification of autophagic vacuoles and regulators of autophagy in villous trophoblast from normal term pregnancies and in fetal growth restriction. *J Matern Fetal Neonatal Med* (2013) 26(4):339–46. doi: 10.3109/14767058.2012.733764
262. Tsukamoto S, Kuma A, Murakami M, Kishi C, Yamamoto A, Mizushima N. Autophagy is essential for preimplantation development of mouse embryos. *Sci (80-)* (2008) 321(5885):117–20. doi: 10.1126/science.1154822
263. Hiyama M, Kusakabe KT, Takeshita A, Sugi S, Kuniyoshi N, Imai H, et al. Nutrient starvation affects expression of LC3 family at the feto-maternal interface during murine placentation. *J Vet Med Sci* (2015) 77(3):305–11. doi: 10.1292/jvms.14-0490
264. Gao L, Qi H-B, Kamana KC, Zhang X-M, Zhang H, Baker PN. Excessive autophagy induces the failure of trophoblast invasion and vasculature: possible relevance to the pathogenesis of preeclampsia. *J Hypertens* (2015) 33(1):106–17. doi: 10.1097/HJH.0000000000000366
265. Oh S-Y, Choi S-J, Kyung Hee Kim KH, Cho E, Kim J-H, Roh C-R. Autophagy-Related Proteins, LC3 and Beclin-1, in Placentas From Pregnancies Complicated by Preeclampsia. *Reprod Sci* (2008) 15(9):912–20. doi: 10.1177/1933719108319159
266. Yang ZJ, Chee CE, Huang S, Sinicrope FA. The role of autophagy in cancer: Therapeutic implications. *Mol Cancer Ther* (2011) 10(9):1533–41. doi: 10.1158/1535-7163.MCT-11-0047
267. Amaravadi R, Kimmelman AC, White E. Recent insights into the function of autophagy in cancer. *Genes Dev* (2016) 30(17):1913–30. doi: 10.1101/gad.287524.116
268. Yang A, Rajeshkumar NV, Wang X, Yabuuchi S, Alexander BM, Chu GC, et al. Autophagy is critical for pancreatic tumor growth and progression in tumors with p53 alterations. *Nature* (2014) 504(7479):296–300. doi: 10.1158/2159-8290.CD-14-0362
269. Santanam U, Banach-Petrosky W, Abate-Shen C, Shen MM, White E, Dipaola RS. Atg7 cooperates with Pten loss to drive prostate cancer tumor growth. *Genes Dev* (2016) 30(4):399–407. doi: 10.1101/gad.274134.115
270. Guo JY, Karsli-Uzunbas G, Mathew R, Aisner SC, Kamphorst JJ, Strohecker AM, et al. Autophagy suppresses progression of K-ras-induced lung tumors to oncocytomas and maintains lipid homeostasis. *Genes Dev* (2013) 27(13):1447–61. doi: 10.1101/gad.219642.113
271. Rao S, Tortola L, Perlot T, Wirnsberger G, Novatchkova M, Nitsch R, et al. A dual role for autophagy in a murine model of lung cancer. *Nat Commun* (2014) 5(1):1–15. doi: 10.1038/ncomms4056
272. Mah LY, Ryan KM. Autophagy and cancer. *Cold Spring Harbor Perspect Biol* (2012) 4(1):a008821. doi: 10.1101/cshperspect.a008821
273. Chung C, Seo W, Silwal P, Jo EK. Crosstalks between inflammasome and autophagy in cancer. *J Hematol Oncol* (2020) 13(1):100. doi: 10.1186/s13045-020-00936-9
274. Slobodnyuk K, Radic N, Ivanova S, Llado A, Trempolec N, Zorzano A, et al. Autophagy-induced senescence is regulated by p38α signaling. *Cell Death Dis* (2019) 10(6):1–18. doi: 10.1038/s41419-019-1607-0
275. Lee S, Schmitt CA. The dynamic nature of senescence in cancer. *Nat Cell Biol* (2019) 21(1):94–101. doi: 10.1038/s41556-018-0249-2
276. Huang T, Song X, Yang Y, Wan X, Alvarez AA, Sastry N, et al. Autophagy and hallmarks of cancer. *Crit Rev Oncog* (2018) 23(5–6):247–67. doi: 10.1615/CritRevOncog.2018027913
277. Singh SS, Vats S, Chia AYQ, Tan TZ, Deng S, Ong MS, et al. Dual role of autophagy in hallmarks of cancer. *Oncogene* (2018) 37(9):1142–58. doi: 10.1038/s41388-017-0046-6
278. Sharifi MN, Mowers EE, Drake LE, Collier C, Chen H, Zamora M, et al. Autophagy Promotes Focal Adhesion Disassembly and Cell Motility of Metastatic Tumor Cells through the Direct Interaction of Paxillin with LC3. *Cell Rep* (2016) 15(8):1660–72. doi: 10.1016/j.celrep.2016.04.065
279. Sarcar B, Li X, Fleming JB. Hypoxia-Induced Autophagy Degrades Stromal Lumican into Tumor Microenvironment of Pancreatic Ductal Adenocarcinoma: A Mini-Review HHS Public Access. *J Cancer Treat Diagn* (2019) 3(1):22–7. doi: 10.29245/2578-2967/2019/1.1165
280. Guo JY, Chen HY, Mathew R, Fan J, Strohecker AM, Karsli-Uzunbas G, et al. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. *Genes Dev* (2011) 25(5):460–70. doi: 10.1101/gad.2016311
281. Lock R, Roy S, Kenific CM, Su JS, Salas E, Ronen SM, et al. Autophagy facilitates glycolysis during Ras-mediated oncogenic transformation. *Mol Biol Cell* (2011) 22(2):165–78. doi: 10.1091/mbc.E10-06-0500
282. Lock R, Kenific CM, Leidal AM, Salas E, Debnath J. Autophagy-dependent production of secreted factors facilitates oncogenic RAS-Driven invasion. *Cancer Discovery* (2014) 4(4):466–79. doi: 10.1158/2159-8290.CD-13-0841
283. Choi AMK, Ryter SW, Levine B. Mechanisms of disease: Autophagy in human health and disease. *N Engl J Med* (2013) 368(7):651–62. doi: 10.1056/NEJMra1205406

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Carvajal, Gutiérrez, Morselli and Leiva. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Oncology

REVIEW
published: 10 June 2021
doi: 10.3389/fonc.2021.652133

A Key Pathway to Cancer Resilience: The Role of Autophagy in Glioblastomas

Elisa Helena Farias Jandrey[1†], *Marcelle Bezerra*[1†], *Lilian Tiemi Inoue*[1], *Frank B. Furnari*[2], *Anamaria Aranha Camargo*[1] *and Érico Tosoni Costa*[1*]

[1] Molecular Oncology Center, Hospital Sírio-Libanês, São Paulo, Brazil, [2] Ludwig Institute for Cancer Research, University of California San Diego (UCSD), San Diego, CA, United States

There are no effective strategies for the successful treatment of glioblastomas (GBM). Current therapeutic modalities effectively target bulk tumor cells but leave behind marginal GBM cells that escape from the surgical margins and radiotherapy field, exhibiting high migratory phenotype and resistance to all available anti-glioma therapies. Drug resistance is mostly driven by tumor cell plasticity: a concept associated with reactivating transcriptional programs in response to adverse and dynamic conditions from the tumor microenvironment. Autophagy, or "self-eating", pathway is an emerging target for cancer therapy and has been regarded as one of the key drivers of cell plasticity in response to energy demanding stress conditions. Many studies shed light on the importance of autophagy as an adaptive mechanism, protecting GBM cells from unfavorable conditions, while others recognize that autophagy can kill those cells by triggering a non-apoptotic cell death program, called 'autophagy cell death' (ACD). In this review, we carefully analyzed literature data and conclude that there is no clear evidence indicating the presence of ACD under pathophysiological settings in GBM disease. It seems to be exclusively induced by excessive (supra-physiological) stress signals, mostly from *in vitro* cell culture studies. Instead, pre-clinical and clinical data indicate that autophagy is an emblematic example of the 'dark-side' of a rescue pathway that contributes profoundly to a pro-tumoral adaptive response. From a standpoint of treating the real human disease, only combinatorial therapy targeting autophagy with cytotoxic drugs in the adjuvant setting for GBM patients, associated with the development of less toxic and more specific autophagy inhibitors, may inhibit adaptive response and enhance the sensibility of glioma cells to conventional therapies.

Keywords: autophagy, glioblastoma, intratumoral heterogeneity (ITH), drug resistance, cell invasion, pro-tumoral

OPEN ACCESS

Edited by:
Eugenia Morselli, Pontificia Universidad Católica de chile, Chile

Reviewed by:
Ilya Ulasov, I.M. Sechenov First Moscow State Medical University, Russia
Gabriele Multhoff, Technical University of Munich, Germany

***Correspondence:**
Érico Tosoni Costa
ecosta@mochsl.org.br

[†]*These authors share first authorship*

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *11 January 2021*
Accepted: *17 May 2021*
Published: *10 June 2021*

Citation:
Jandrey EHF, Bezerra M, Inoue LT, Furnari FB, Camargo AA and Costa ET (2021) A Key Pathway to Cancer Resilience: The Role of Autophagy in Glioblastomas. Front. Oncol. 11:652133. doi: 10.3389/fonc.2021.652133

INTRODUCTION

Glioblastoma (GBM, grade IV astrocytoma) is the most frequent, life-threatening malignant brain tumor and one of the most resilient of all human malignancies. Those tumors are classified and subtyped based on histopathological traits, clinical presentation, and molecular status (1). The current treatment for GBM includes gross neurosurgical resection with the oral use of alkylating

agent temozolomide (TMZ), which is given concurrently with radiotherapy (RT) and as an adjuvant monotherapy. Despite aggressive treatments, patients have a low median survival of ~12 months (2–4).

One of the key factors in GBM's aggressiveness and resilience is their high cell plasticity: a concept associated with phenotype switching, based on the reactivation of transcriptional programs related to the acquisition stem cell properties and the migratory phenotype (5). In the context of anti-glioma therapies, cell plasticity enables tumor cells to change to a cell phenotypic identity, enabling them to survive the dynamic changes of the tumor microenvironment (TME) and to escape surgery and radiotherapy margins by migration. A remarkable example of this plasticity in GBM cells was conceptualized in the "go-or-grow" dichotomous concept in gliomas. It is based on the notion that phenotypically distinct GBM cells (at the "go" or "grow" states) coexist and cooperate to promote tumor growth and clinical relapse: chemoradiation effectively eliminates the bulk population of highly proliferative cells (at the "grow" state), leaving behind a subpopulation of dormant/migratory cells (at the "go" state). "Go" and "grow" states are completely reversible insofar as GBM cells change their phenotypes without genetic mutations. This plasticity is controlled by different signaling pathways that drive adaptive responses and emerge as a non-genetic source of functional intratumoral heterogeneity that, ultimately, mirror tumor resiliency and high patient mortality (2, 6, 7).

Autophagy (greek "self-eating") is a good example of signaling pathway associated with the phenotype switching and metabolic flexibility of GBM cells. It is primarily a degradative pathway characterized as a fast route by which damaged cytoplasmic materials (collectively named 'cargo') are delivered to the lysosomes for recycling. Autophagy can be categorized into 3 subtypes called, micro-autophagy, macro-autophagy, and chaperone-mediated autophagy (for more detailed insights into the different autophagic pathways see (8–10).

Macroautophagy (hereafter referred to as autophagy) must take place on a baseline in each cell to withdraw damaged and functionless organelles, providing metabolites to synthetic pathways and sustaining energetic homeostasis. In the brain, baseline autophagy is important as a clearance mechanism of disease-related proteins in neurons and also in astrocytes, and autophagy dysfunction may contribute to the progression of neurodegenerative diseases (11). However, a selective activation of autophagy can be observed in various pathophysiological and/or stress situations (12–15). For example, in normal brain Beclin-1 (BECN1), a gene with a central role in autophagy induction (16), was not expressed by neurons or glial cells, but showed strong cytoplasmic overexpression in primary GBM cells (17). Moreover, in response to standard of care in patients with GBM (radio- and chemotherapy), the autophagy pathway is upregulated giving tumor cells an advantage for survival. In a series of clinicopathological studies, cancer cells exhibit an increased autophagy activity linked with poor prognosis and aggressive clinical behavior (17–19). Those are emblematic examples of the 'dark-side' of autophagy, acting as a therapy-responsive mechanism associated with a pro-tumoral adaptive response (20–25).

On the other hand, there are numerous reports, mostly from *in vitro* cell-based studies, showing an anti-tumoral function of autophagy. Those reports have clearly shown that excessive activation of the autophagy by prolonged or supraphysiological doses of stress signals, may lead to massive removal of cytosolic material, leading to a specific type of non-apoptotic cell death, named type II programmed cell death, or autophagic cell death (ACD). ACD is characterized by large-scale autophagic vacuolization of the cytoplasm in the absence of chromatin condensation and can be specifically blocked by the inhibition of autophagy-related genes (ATG) (26, 27). Due to this 'dual' role in human cancers cells, autophagy is, therefore, often been described metaphorically as a 'double-edged sword' in cancers. Importantly, the studies that explore the mechanisms of ACD are mostly from *in vitro* cell-based approaches, which provide us a precious source of mechanistic insights, but are of limited translational relevance. Of note, there is no doubt that GBM cells activate autophagy shortly before or during their death in according to the external cues or internal stimuli received, but it is still controversial whether this activation contributes to cell death or rather represents a last attempt of survival.

So, to understand the real effect of autophagy in GBM disease is necessary to analyze cancer cells under normal pathophysiological conditions and therapeutic doses. In the next sections, we will focus on the specific extracellular signals that surround tumors and play an important role in controlling autophagy in GBM cells. Important is the notion that our particular emphasis was given to studies that evaluate the relationship between autophagy and GBM from a perspective of understanding and treating human disease. Therefore, studies using *in vitro* cell-based models, inducing ACD by excessive stress signals, were not fully considered here, except for the mechanistic data.

AUTOPHAGY ACTIVATION AS A RESPONSE TO PATHOPHYSIOLOGICAL STRESS

Necrosis and acidic stress are the most important stress signals in GBM microenvironment related with autophagy activation. Tumor necrosis is a histological hallmark of grade IV astrocytic tumors with prevalence in almost 90% of patients with GBM (1, 28, 29). Necrosis appears as either multifocal areas (micronecrosis) or broad necrotic areas surrounded by hyperproliferative zones of tumor cells, called perinecrotic niches (PNN), which is visible as a soft, gray rim surrounding necrotic areas by magnetic resonance imaging (MRI). During disease progression and treatment response, GBM cells have to change their metabolism to survive in PNN, characterized by intermittent hypoxia (defined by low oxygen levels, $pO_2 < 3\%$) and starvation conditions due to poor functional vasculature (30, 31). This configuration is indirectly linked to poor patient outcome and associated with radio and TMZ resistance (32, 33). Hypoxia, *per se*, is well known to create radiation and

chemotherapy resistances (34). As part of the physiological adaptive response, the PNN stimulates the stabilization of hypoxia-induced factors (HIFs), HIF1a and HIF2a, resulting in a driving force for activation of anti-apoptotic and pro-migratory transcriptional programs, supporting angiogenesis (35, 36), and re-expression of markers and properties typical of glioma stem cells (GSCs) (30, 37–42). Interestingly, hypoxia, starvation and conventional anti-glioma therapies stimulate the onset of autophagy above baseline levels in GBM cells.

Hypoxic conditions also shift GBM cells towards aerobic glycolysis, rather than mitochondrial oxidative phosphorylation, promoting an acidic environment, potentially favoring tumor invasion by pH-dependent activation of proteinases (e.g. heparanases and cathepsins) (43). Heparanase (HPSE) is an endo-β-D-glucuronidase that has both enzymatic and non-enzymatic functionalities in a pH-dependent manner. HPSE expression is intrinsically correlated with GBM progression, worse prognosis (44), and cell invasion (45). Intriguingly, autophagy is one of the cellular mechanisms regulated by heparanase activity in various tumors, including brain tumors (46). Notably, autophagy induced by starvation in GBM cells was prevented by the use of a potent heparanase inhibitor. Moreover, in these cells the pro-tumorigenic function of heparanase is mediated by autophagy activation, enhancing chemotherapy resistance in nutrition-stressed environments. The mechanism underlying heparanase-induced autophagy is not fully understood but appears to involve mTOR1 inhibition, which plays a pivotal role in nutrient-sensing and autophagy regulation *in vitro* (47).

Cathepsins belong to a class of cysteine proteinases that is mainly expressed by GSC subpopulations of IDH wild-type GBM patients (48). Cathepsins can be secreted into the extracellular space and have an optimum activity on acidic environments to further activate MMP proenzymes (49), with have an important role in controlling tumor cell invasion, stem cell phenotypes (50–53) and tumor progression (54). Cathepsin D levels, for example, are strongly and positively correlated with LC3A and LC3B expression in GBM patients (markers for autophagosome levels) (17). Moreover, inhibition of Cathepsin D attenuates autophagy, leading to increased radiosensitivity in GBM cells. In radioresistant cells, Cathepsin D has been positively correlated with LC3-II and negatively correlated with p62 (55), a protein that targets specific cargoes for autophagy (56). The expression levels of another member of family, the Cathepsin L, are higher in GBM compared to low-grade gliomas (57), exerting an important role in migratory phenotype (51, 52, 58, 59) and γ-radiation-induced GBM cell invasion (59). Interestingly, autophagy inhibition by trifluoperazine induces radiosensitivity in GBM cells mediated by Cathepsin L downregulation (60).

Interestingly, at PNN (i.e. under physiological hypoxia), autophagy activation via BNIP3/BNIP3L is a survival mechanism that promotes GBM progression and resistance to anticancer therapies *in vivo* (61). Recently, a global analysis conducted by Bronisz et al. that included 41 GBM patient's cohort identified the autophagy pathway as the unique de-regulated pathway in PNNs of primary GBMs (32). These analyses indicate that poorly perfused tumor regions are likely to have increased baseline autophagic levels and, therefore, under hypoxic conditions, the increased autophagic flux may play an adaptive role (62, 63). Under hypoxia, autophagy is activated by BECN1 phosphorylation *via* the HIF-1a/BECN1 signaling pathway, one of the initial steps in the assembly of autophagosomes from pre-autophagic structures (64–66). Moreover, PNN in GBM disease also show the overexpression of interleukin 6 (IL6), an inflammatory cytokine that is essential for hypoxia-induced autophagy and induction of invasive programs in GBM cells (67–71). At this point, is important to notice that under *in vitro* prolonged hypoxic stress (48-72h, <1% pO_2), the gene BNIP3 (Bcl-2/adenovirus E1B 19kDa-interacting protein 3), a pro-apoptotic Bcl-2 family member, is upregulated, leading to hypoxia-dependent ACD in GBM cells (72). Mechanistically, BNIP3 upregulation releases BECN1 from the complexes with Bcl-2 or Bcl-xL, allowing BECN1 to activate autophagy (73). It becomes especially critical to note that the nature of the autophagic response to hypoxia - a cytoprotective or cytotoxic output - depends on the extent and duration of the microenvironmental stressor, on the experimental design, as well as, on the genetic background of the tumor cells.

Alternative forms to GBM cells to adapt or to avoid poor oxygenation and hostile microenvironment are through the vasculogenic mimicry (VM) phenomenon (74) and the activation of migratory programs by altering the composition of the TME (75–77). VM represents an impressive example of a higher phenotype flexibility of GBM cells. GBM cells capable of VM formation organize themselves into functional vascular-like structures, ensuring tumor blood supply independently of normal blood vessels or angiogenesis. In this scenario, it has been shown that VM formation in glioma patients was associated with the expression of BECN1 (16).

Moreover, as a part of adaptive programs, VM formation is also promoted by Bevacizumab (BVZ)-induced autophagy in GSC, an anti-VEGF antibody that received accelerated approval by the FDA to treat recurrent GBM (78), which is associated with tumor resistance to antiangiogenic therapy (see below) (79). VM was also associated with high expression of HIF-1α (80) and upregulation of the IL-8/CXCR2 pathway (81). It is also conceivable that autophagy may contribute to the increased production of multiple pro-invasive cytokines, including interleukin-6 (IL-6) and -8 (IL-8), which, in turn, may reactivate a pro-invasive and GSC transcriptional programs, leading GBM cells to the "go" state, allowing them to migrate away from cytotoxic niches towards a supportive microenvironment (69, 82).

Decorin (DCN), a member of the small leucine-rich proteoglycans (PGs) family, has a vital role in the hypoxia-dependent activation of autophagy and anti-glioma therapy resistance, mainly due to their binding to VEGFR2 expressed by vECs, particularly in PNNs of glioma samples, or with the binding to c-Met and EGFR receptors expressed by GBM cells (83, 84). High levels of c-Met or DCN correlate with shorter progression-free survival (PFS) and overall survival (OS) in

patients with GBM (85–88). The high-affinity DCN/receptor interaction leads to increased expression of paternally expressed gene 3 (Peg3), that physically associates with BECN1, recruiting LC3 into autophagosomes (89, 90). Complementarily, in GSC-enriched environments, GBM cells produce a high amount of PGs, such as DCN and Lumican, promoting chemotherapy resistance and cell survival (91). Curiously, as observed in several types of non-central nervous system tumors (92, 93), soluble DCN potently induces autophagy in GBM cells and contributes to an impairment of GBM cell migration *in vitro* experiments (94). Other extracellular matrix (ECM) proteins, such as endostatin, perlecan, and endorepellin, can influence tumor progression by regulating autophagy levels in endothelial cells, controlling vessel formation and neo-angiogenesis in response to hypoxia (95, 96).

AUTOPHAGY ACTIVATION AS A RESPONSE TO PHYSIOLOGICAL SIGNALS

Once PNN and other stress signals reactivate migration programs to drive plasticity and invasiveness in GBM cells, invasive growth along specific anatomical structures, especially at the vasculature and white matter tracts, is regarded as the main cause of poor therapeutic outcome of patients with GBMs. The migration occurs at perivascular niches (PVN), besides PNN, and considered the preferred and fastest route for GBM cell invasion through brain tissue (97). PVNs are fluid-filled spaces, continuous to the subarachnoid space, surrounding all blood vessels in the brain, including capillaries and arterioles. Based on histological information and *in situ* experiments, a widely accepted idea is that GBM cells actively seek out PVNs and migrate along with them (98, 99). For example, bradykinin, produced by cerebral vascular endothelial cells (vEC), acts as a strong chemotactic signaling peptide, guiding GBM cells toward PVN. Therefore, when injected into mice brain, the vast majority (over 85%) of human GBM cells move into contact with a blood vessel (100). At PVN, cerebral vECs are in the closest proximity to tumor cells. This heterotypic interaction induces a GSC transdifferentiation, which is critical for the malignant traits of the disease and supports the notion that stemness is a temporary reversible trait of GBM cells. The GSC phenotype is maintained by vECs *via* mediators, such as nitric oxide (NO), cyclic guanosine monophosphate (cGMP), and Notch1 ligands (97, 101–104). The stemness phenotype has been recently associated to autophagy activation and is one of the most important processes in the PVN responsible for the maintenance of GSC status besides PNN (105, 106). Additionally, the interaction between GBM cells and pericytes at PVN leads to chaperone-mediated autophagy in normal pericytes, building an immunosuppressed microenvironment that induces GSC phenotype and tumor growth (107). Interestingly, activation of protective autophagy in cerebral vECs is one of the essential physiological processes responsible for maintaining vascular homeostasis, and playing an important role in vECs proliferation, migration, and tube formation (108, 109). Other types of vEC-derived molecules also promote autophagy and correlate with stemness in GBM cells. For example, osteopontin (OPN), derived from the vEC, plays an oncogenic role and initiates a stem-promoting cascade and enhances autophagy through an integrin-CD44 dependent activation of HIF genes at PVNs (110, 111). OPN-elicited autophagy could promote cancer cell survival, resistance to chemotherapy drugs, and has been associated with increased glioma grade and migratory potential (112).

The melanoma-differentiation associated protein 9 (MDA-9, also called Syntenin-1) is another ECM protein that sponsors tumor invasion mainly by regulating the cell surface receptor Syndecan (113). In GBM, MDA-9 expression is an important regulator of cell invasion (114), stemness phenotype, and survival of GSCs through STAT3 and Notch1 pathways, respectively (115). Interestingly, the MDA-9 is responsible for activating protective autophagy in GSCs *in vitro* through the EGFR/FAK and EGFR/PKC axis, inhibiting anoikis (a suspension-induced form of apoptosis) by the hyperphosphorylation of Bcl-2 (116). In this scenario, autophagy often is activated in these cells as a compensatory pro-survival adaptation to detachment stress. In such cases, autophagy precedes (and usually avoids) anoikis by removing pro-apoptotic proteins in the cytosol. For example, depletion of ATG5 or ATG7 inhibits detachment-induced autophagy and enhances anoikis (117, 118). A higher expression of MDA-9 has been linked to higher glioma grade and short-term survival (119).

AUTOPHAGY ACTIVATION AS A RESPONSE TO ANTI-GLIOMA THERAPIES

RT plus concomitant and maintenance TMZ is the gold standard treatment and represent a major advance in the field of therapy for high-grade gliomas (7, 120). The addition of BVZ to standard treatment revealed an improvement in progression-free interval but had no effect on OS (121). Intriguingly, virtually all glioma therapies, including RT, TMZ and/or BVZ, are stronger inducers of autophagy pathway: several pre-clinical and clinicopathological studies indicate that increased autophagy activity help to desensitize GBM cells to treatment and it is linked with poor prognosis in different cancers (21). Inversely, others observations shown that excessive intensification of autophagic process lead to cell exhaustion and death (26, 62, 72, 122). So, despite the potential 'dual' role of autophagy has been clearly observed in cell-based studies, in 'real' disease, the predominant data conduct to the idea that therapy-induced autophagy is acting as an adaptive response and a protective mechanism in GBM cells instead of eliciting cell death.

The study of Natsumeda et al. (2011) is probably the first to show the induction of autophagy by TMZ in glioma cells and in reactive astrocytes of glioma patients by immunohistochemical analysis, indicating some type of stress response in tumor and normal cells (22). The addition of chloroquine (CQ) and its derivative hydroxychloroquine (HCQ) – both inhibitors of

autophagy by blocking autophagosome fusion and degradation - to TMZ-treated glioma cells attenuates autophagy flux, induces accumulation of the proautophagy proteins (LC3-II) and promotes endoplasmic reticulum stress and cleavage of PARP (a marker of apoptosis) (123). Many other studies observed that blocking autophagosome formation enhances TMZ cytotoxicity, indicating that the autophagy pathway may protect GBM cells from TMZ-induced cytotoxicity (25, 123). For example, it has been demonstrated that CQ plus TMZ significantly increased the amounts of cleaved PARP (a marker for apoptosis) over those cells treated with TMZ alone. The pharmacological inhibition of autophagy by CQ also negatively dictates the migratory capacity of GBM cells, corroborating the role of autophagy with other aspects of adaptive phenotype and cell plasticity (124). While other authors have suggested that autophagy is the main component of TMZ-induced cytotoxicity and that inhibition of the autophagy significantly influences the antitumor effect of TMZ *in vitro* (20).

Ionizing radiation is the gold-standard adjuvant treatment for GBM. Radiotherapy also results in enhanced autophagy in GBM cells *in vitro* (125). When irradiated, many GBM cells undergo cell death by apoptosis, whereas GBM cells that do not undergo apoptosis activate autophagy, suggesting a protective mechanism (24, 125).

It has also been demonstrated that CQ treatments can increase radiosensitivity in GBM cells (25). Moreover, CQ worked synergistically with radiotherapy for induction of apoptosis in GSC; thereby acting as a protective mechanism (126). Another study showed that DNA-protein kinase-deficient GBM cells (DNA-PK), an enzyme that plays a critical role in DNA double-strand breaks repair, underwent massive ACD even after low doses of γ-radiation in cell lines *in vitro*. Intact DNA-PK pathway prevented ACD, but cells still exhibited a low apoptotic tendency, indicating that genetic background takes a leading role on the sensitivity of treatment and cell fate determination (127).

Another example of therapy-induced autophagy occurs after the use of antiangiogenic therapies in GBM. The addition of BVZ to conventional chemoradiation improved the PFS but did not affect OS (121). At the TME level, BVZ induces a hypoxic niche that results in protective autophagy sponsoring GBM cell resistance and survival. Alternatively, BVZ induced autophagy directly in GBM cells by suppressing the Akt-mTOR signaling pathway (128). Furthermore, BVZ-mediated autophagy is also dependent on interferon regulatory factor 1 (IRF1) expression in gliomas (129). Moreover, GBM cells expressing the stem cell markers CD133 and Sox2, and residing in the PVN, internalize BVZ through micropinocytosis, leading to autophagy activation and cell survival (130). Autophagy inhibition by ATG7 silencing rescued GBM sensitivity to BVZ treatments (131).

AUTOPHAGY ACTIVATION AS A RESPONSE TO INTERNAL STIMULI

Autophagy in GBM cells is triggered in response to external or internal stimuli. Internal stimuli is manifested directly by alterations in ATG or indirectly by oncogenic proteins commonly found aberrantly expressed in GBM and lower-grade gliomas. The following subsections cover the most important genetic events for gliomagenesis and their specific genetic aberrations associated with autophagy activation.

There are 16 known ATG in humans, four of which (ATG2B, ATG5, ATG9B and ATG12) are frequently mutated in gastric and colorectal cancers, and in hepatocellular carcinoma, and may be causally associated with cancer development by deregulating the autophagy process (132, 133). Large-scale genomic analysis indicates that core autophagy genes are generally not mutated in patients of 11 human cancers, including GBM, suggesting that the autophagy machinery is functional in cancer types investigated (134, 135). At a clinical perspective, several ATG signatures have been emerging as important prognostic factors for GBM patients, and autophagy high scores have been related to worse outcomes (136–138). For example, Wang and colleagues described that a robust 14-mRNA prognostic signature was an independent prognostic factor associated with OS in GBM's patients (HR=1.9, 95% CI = 1.013-3.644, p value = 0.045) (136). Moreover, several other research groups have correlated the higher expression of ATGs with glioma aggressiveness, including patient's poor survival and tumor progression (139–143). Despite their prognostic relevance, for future clinical applications, it is also important to integrate with other types of signatures (such as protein signatures).

Large-scale genomic studies showed that primary GBM arises from defects in three main molecular signaling pathways involving p53, Rb, and phosphoinositide 3-kinase (PI3K) (144). The phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) cascade is recognized as an important sensor of nutrient/growth factor availability and a major pathway regulating autophagy in human cancers. In a permissive microenvironment, active PI3K/Akt/mTOR cascade constitutively suppresses autophagosomes biogenesis by inactivating the ATG1/ULK1 complex or by sequestration and inactivation of BECN1, both considered key initiators of the autophagic pathway (145, 146). Inhibitors of Akt/mTOR activity, such as rapamycin analogs, intensify the autophagic process (147). However, under stressful conditions, PI3K/Akt/mTOR cascade is normally inactivated through extracellular signals, like intermittent hypoxia and depletion of nutrients, leading to the extrinsic activation of protective autophagy. Nevertheless, in GBM samples, activation of PI3K/Akt/mTOR cascade is observed in almost 90% of the cases, and caused by the overexpression of upstream activators, like epidermal growth factor receptor (EGFR) or c-Met, activating mutations of PI3CA (p110) or PIK3R1 (P85) (148–150), and inactivating mutations in the phosphatase and tensin homolog (PTEN), a negative regulator of PI3K activity (loss-of-function mutations in PTEN are present in almost 60-85% of GBMs) (151, 152). Moreover, the use of a potent PI3K inhibitor promotes autophagy activation at the expense of invasion and angiogenesis impairment in GBM cells. Furthermore, PI3K inhibition also restrained tumor growth and significantly prolonged mouse survival (153). In addition,

GBM cells harboring mTOR hyper-activation, showed an increment of autophagy after the use of rapamycin (154).

The deregulation of the tumor protein p53 (TP53) pathway accounts for approximately 85% of GBMs, including alterations on CDKN2A, MDM2 and TP53 genes (155). Members of this signaling pathway have been described as modulators of migration, invasion, proliferation, and stemness, leading to poor prognosis in GBM patients (155). Regarding autophagy activation, nuclear p53 induces the expression of the ATGs: DRAM, and Sestrins 1/2. Indeed, DRAM1 is considered the regulator of the autophagy activation mediated by nuclear p53 (156–158), promoting migration and invasion of glioma stem cells (141). Interestingly, cytoplasmic p53 inhibits autophagy, but external stressors, such as nutrition starvation, induces the destruction of cytoplasmic p53, sustaining autophagy activation (159, 160). More recently, it has been shown that combined therapy with TMZ and CQ synergistically reduces cell proliferation and enhances apoptosis in p53-wild type cells. Overexpression of mutant p53 abolishes the autophagic vacuoles (161).

The Retinoblastoma gene (RB1) is a tumor suppressor gene commonly mutated or deleted in GBM and correlated with lower survival rates in astrocytomas patients (162, 163). Functionally, Rb inhibits cell cycle progression and promotes cell survival by controlling the function of the E2F transcription factor (164). Besides cell cycle transition control, Rb also influences tumor cell differentiation, senescence, apoptosis, and autophagy (165). Indeed, Rb downstream effector E2F1 directly mediates the expression of the autophagy-related genes LC3, ATG1, and DRAM (166). In GBM cells, it has been shown that Rb binds to E2F, repressing its activity, and leading to autophagy induction. Indeed, Rb activity or E2F1 silencing induced autophagic flux through increased autophagosome formation (167). Interestingly, while the binding of Rb to E2F promotes the activation of autophagy, Rb phosphorylation represses its binding to E2F and leads to apoptosis activation (168). In this scenario, it has already been shown that the Rb-E2F axis regulates the expression of the BNIP3 gene, an essential gene that mediates hypoxia-induced autophagy, promoting autophagosome formation in nutrient-deficient environments (169). Rb-induced autophagy is considered a resistance mechanism in GBM cells treated with etoposide or cisplatin (170, 171).

The most relevant and frequent oncogenic alterations in GBM patients involve the Epidermal growth factor receptor (EGFR), comprising 57% of patients. These alterations include mutations, rearrangements, amplifications, and splicing variants that lead to enhanced tumor growth, angiogenesis, survival, and stemness (148, 172). Intriguingly, due to the functional impact of EGFR alterations on tumor aggressiveness, lower- grade gliomas harboring EGFR amplification are considered "GBM-like tumors" due their aggressive phenotypic behavior (173). Beyond the known pathological role of EGFR on GBMs, their functions in autophagy regulation are emerging, indicating that it directly acts as a controller of the autophagic flux by mTOR signaling modulation (174, 175). EGFR-mediated autophagy exerts relevant roles in gliomagenesis, tumor progression, and therapy resistance (176). Clinically, GBM patients with low levels of EGFR and high expression of BECN1 have a median overall survival of 30 months, presenting a favorable response to radiotherapy (177). Therapeutically, the combination of tyrosine kinase inhibitors (TKI), such as erlotinib, with CQ increases the antineoplastic effect of the TKI on apoptosis-resistant GBM cells (178). Surprisingly, another EGFR inhibitor, called BIBU, impaired Akt and STAT3 activation, induced apoptosis death, and activated protective autophagy (179). The constitutively active mutant allele of EGFR, known as EGFRvIII is an important mediator of autophagy (180). It occurs in 20–30% of all human GBM, making it the most common EGFR mutant in GBM (181, 182). EGFRvIII-expressing GBMs are intrinsically resistant to apoptosis induced by radio- and chemotherapy (183, 184). Interestingly, these tumors have autophagy over-activation under hypoxic conditions and patients benefit from the use of CQ (180). Intriguingly, GBM cells harboring EGFRvIII alterations are more sensitive to the pharmacological inhibition of mTOR (185).

c-MET (also called HGFR) is a type of Receptor Tyrosine Kinase mutated in 6% and amplified in 4% of patients with GBM, leading to constitutive activity. Patients harboring c-MET gain-of-function alterations present a shorter survival and poor response to treatment (186). The enhancement of c-Met activity induces GBM cell survival, proliferation, invasion, angiogenesis, and stemness (187). The intracellular pathway triggered by c-MET is PI3K/Akt signaling. Additionally, cell invasion mediated by c-MET relies on Focal Adhesion Kinase (FAK) activity (188). Interestingly, c-MET expression was correlated with autophagy activation in GSCs, positively regulating their migratory and invasive capacity (141). c-MET expression abrogation by epigenetic silencing in glioma cells suppresses Akt pathway activation and up-regulates the expression of the autophagy-related protein Atg5, resulting in tumor growth reduction (189).

Isocitrate dehydrogenase 1 and 2 (IDH1/2) mutations are the most important molecular markers in diffuse gliomas due to their high impact on patient survival improvement and tumor development (190, 191). IDH1 mutations (IDH^{mut}) are present in more than 80% of low-grade gliomas (grades II-III) and in secondary GBMs, but are rare in primary GBMs (190, 192). Mutations in IDH2 have been found in fewer than 3% of glial tumors. Patients with lower-grade gliomas (grades II-III) and glioblastoma show significantly longer OS in the presence of IDH1 or IDH2 mutations (192). The prognostic importance of IDH mutation is independent of other known prognostic factors, including age, grade, and MGMT methylation status. IDH mutations promote a metabolic reprogramming mainly due to the accumulation of the oncometabolite 2- hydroxyglutarate (2-HG), which, in turn, induces the epigenetic silencing of several genes from the glycolytic pathway (193, 194). Moreover, $IDH1^{mut}$ is associated with a distinct hypoxia/angiogenesis transcriptome signature and stabilization of HIF-1a levels in glioma cells (195), important autophagy regulators (see above). Recently, four different groups identified distinct autophagy

signatures with prognostic value in GBMs. High autophagy risk signatures were correlated with patients' worse outcomes. Besides the absence of gene intersection between the signatures, all four achieve the same results: patients with IDH^{mut} tumors presented a lower autophagy-related risk signature compared to IDH wild-type (IDH^{wt}) gliomas, denoting an increased autophagy activation in IDH^{wt} GBMs (136, 137, 196, 197). In the same direction, beyond the gene signatures, it has been shown that higher expression levels of the ATG proteins: LC3, Beclin-1, and p62 are more prevalent in IDH^{wt} gliomas in than IDH^{mut} gliomas (139).

Promoter methylation of the O-6-Methylguanine-DNA Methyltransferase (MGMT) gene is a prognostic marker in patients with glioma because MGMT methylation leads to better response to alkylating agents, such as TMZ (198). Indeed, patients harboring MGMT-methylated GBMs had a 10-month and 4-month higher median overall and PFS, respectively, compared with MGMT-non-methylated patients (199). Interestingly, two different groups showed that MGMT-methylated gliomas presented a lower autophagy risk score compared with MGMT-non-methylated patients (136, 137). In agreement with these data, GBM cell lines that naturally do not express MGMT, highly activate autophagy after TMZ treatment. However, when cells were stably transfected with MGMT, the number of autophagic vacuoles was abrogated after TMZ treatment (122).

Finally, beyond the role of oncogenes, tumor suppressor genes and their downstream signaling molecules, the control of autophagy activation in GBM also relies on the signaling pathways involved in stemness (200). These pathways are mainly involved in the acquisition and maintenance of the GSC phenotype, including Notch, Wnt/β-catenin, and Hedgehog pathways. In gliomas, the activation of *Notch signaling* correlates with more aggressive tumor phenotypes (201). Besides activation of the Notch pathway, its members and ligands are rarely mutated in GBMs (202). The connection between the Notch pathway and autophagy was first described in U87MG and U251 GBM cell lines. When the Notch1 receptor was genetically silenced in these cells, they showed reduced proliferation and viability. GBM growth impairment was correlated with the augmented expression of the autophagy-related proteins Beclin-1 and LC3-II in NOTCH1-silenced cells (203). Complementarily, when autophagy was induced in GSCs by mTOR inhibition, the Notch1 receptor was degraded. Indeed, the impairment of Notch1 signaling induced by autophagy activation led to a decreased tumorigenicity and self-renewal capacity of GSCs (204). Interestingly, the degradation of Notch1 by autophagy is mediated *via* autophagosome-precursor vesicles positively expressing the autophagy-related protein ATG16L1 (205), and by the direct binding of p62 to Notch1 Intracellular Domain (NICD) (206). In contrast, the pharmacological blockage of Notch1 induces cytoprotective autophagy in GBM cells. However, when these cells were exposed to the combination of Notch1 and autophagy inhibitors, treatment resistance was overcome, thus augmenting apoptotic cell death (207). Interestingly, Notch1 signals can be regulated by autophagy activation *via* ATG16L1-positive autophagosomes, modulating stem cell development, and neurogenesis (205).

Wnt signaling plays a critical role in GSC phenotype, therapeutic resistance and invasiveness (208, 209). Mutations in the members of Wnt signaling pathway are not common, but epigenetic alterations are frequently observed in GBMs (210). The inhibition of Wnt signaling by the IWR-1 inhibitor leads to an augment of the expression of the autophagy-related proteins, LC3-II, and Beclin-1 (211). Complementarily, another group showed that the silencing of the intracellular players of Wnt signaling, TCF4, and CTNNB1/β-catenin, induced the up-regulation of SQSTM1/p62, increasing the autophagy flux. Interestingly, Wnt pathway inhibition sensitizes GBM cells to autophagy inhibition with CQ (212). Regarding chemotherapy response to TMZ, the blocking of the Wnt/β -catenin pathway by the activity of the DAB2IP protein is responsible for TMZ resistance through the expression of the autophagy-related protein ATG9B. Interestingly, the combination of TMZ with a Wnt signaling inhibitor can overcome this resistance (213). Indeed, autophagy activation mediated by nutrient starvation in GBM cells down-regulates several mediators Wnt signaling, including activated β-catenin (214).

Hedgehog (Hh) signaling enhances the migratory and invasive capacity of cells through the activation of PI3K/Akt pathway in GBM cells (215). Moreover, it stimulates the growth and tumorigenicity of gliomas, mainly by controlling stemness status (216). Besides the lack of mutations on Hh pathway components in GBMs, it has been shown that the glioma-associated oncogene homolog 1 (GLI1) zinc-finger transcription factors, terminal effectors of the Hh pathway, presents two tumor-specific splicing isoforms, which directly influences tumor malignancy (217). Intriguingly, the activation of Hh signaling is correlated with the modulation of autophagy in several cancer types. Indeed, the inhibition of the Hh pathway negatively controls tumor proliferation by activating autophagy (218). In GBM cells, the use of GANT-61, a specific inhibitor of Gli1 and Gli2, activates autophagy, inducing LC3-II expression, and by negatively modulating the expression of stemness markers and tumor proliferation (219). Pharmacologically, the use of GANT-61 enhances the cytotoxic effect of TMZ by the increment of acid vesicles and Beclin-1 expression (220). Furthermore, the regulatory domain of PTCH1, the main receptor of the Hedgehog pathway, interacts physically with the autophagy-related protein ATG101 in a nutrient starvation microenvironment, inhibiting the autophagic process (221). Additionally, GBM cells overexpressing the stem marker SOX3 showed an upregulation in Hh pathway activity and suppression of autophagy, leading to an increment in proliferation and invasion (222).

THE LAST FRONTIER: THE THERAPEUTIC POTENTIAL OF AUTOPHAGY INHIBITORS FOR THE TREATMENT OF GBM

GBM retains a poor prognostic value and remains incurable. Despite our growing understanding of the mechanisms underlying drug resistance, the standard therapy has not

changed over the last 16 years (7). Up to date, no new therapies improve OS when added to standard therapy, with an exception for the recent Tumor Treating Fields (TTFields) in GBM (223). As we see above, under pathophysiological circumstances, autophagy is a key driver of GBM resistance, allowing cellular adaptive survival towards extrinsic (e.g. hypoxia, drugs or ionizing radiation) or intrinsic (genetic aberrations) stress stimuli (**Figure 1**). Accordingly, from a standpoint of treating GBM disease, targeting autophagy emerge as a new potential therapy and it has been considered a potential candidate to improve the treatment of patients with GBM (224, 225). In this scenario, the use of the autophagy inhibitors, such as CQ or HCQ, has been explored in clinical studies. Those trials mainly focused on the therapeutic potential of autophagy inhibition combined to standard therapies for GBM patients.

A small phase III trial observed a median overall survival of 24 months for patients treated with CQ plus conventional therapy (i.e. surgery, radiotherapy, and carmustine-based chemotherapy) compared to 11 months for patients treated with conventional therapy (226). In a single institutional study with 123 patients, the same authors showed that the addition of CQ to surgery, radiotherapy, and carmustine-based chemotherapy consistently exerts an adjuvant effect, adding more than 13 months in patients' median survival in comparison to control patients (227). However, despite the favorable results in GBM patients treated with CQ combined to surgery, radiotherapy, and carmustine, a phase I/II trial combining HCQ with radiotherapy and TMZ-based chemotherapy showed that the maximum tolerated dose of HCQ was unable to consistently inhibit autophagy and showed no improvement in patient OS (228). To transpose those issues, a new phase I/II trial (NCT02432417) was designed to compare patients treated with concurrent ionizing radiation and TMZ- based chemotherapy with patients treated with this combination plus a most appropriate CQ dose. Indeed, recent data published by the group showed that 200 mg of CQ is a feasible dose to use in those patients, since 400 mg of CQ induced several severe adverse events. Moreover, preliminary data analysis showed an improvement of more than 9 months in the OS of GBM patients harboring EGFRvIII alterations compared with patients without this genetic variant (229). The International Cooperative Phase III Trial is an active clinical trial that evaluates the use of CQ or Valproic acid as an adjuvant to conventional therapy in high-grade gliomas (NCT03243461).

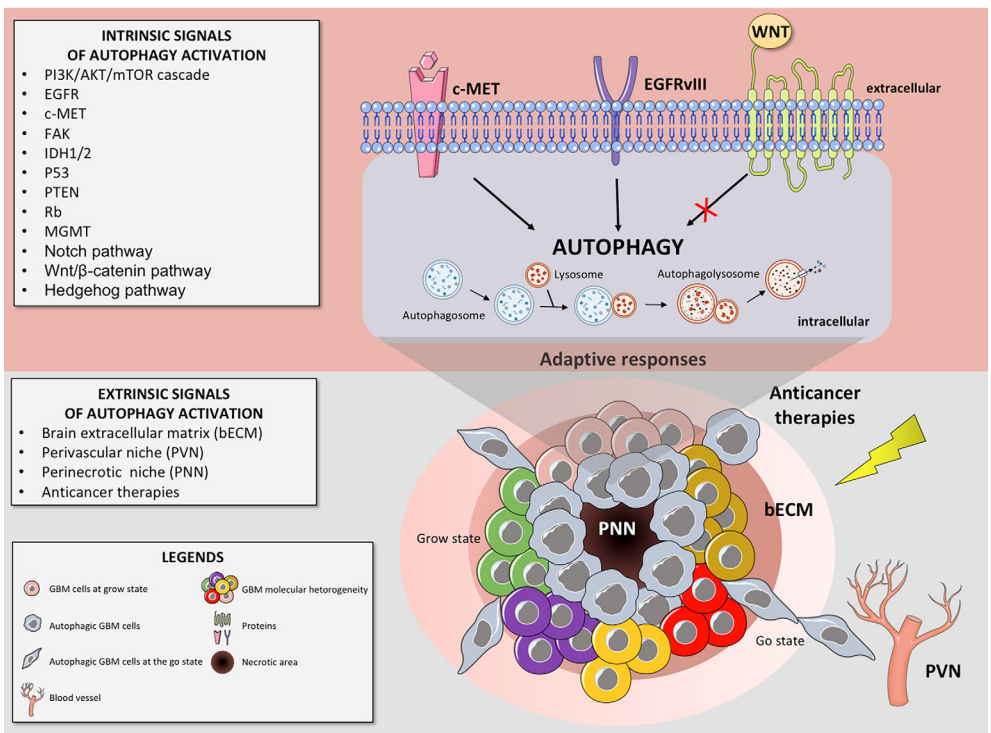


FIGURE 1 | Autophagy can be triggered by intrinsic and/or extrinsic GBM cells signals, contributing to tumor cell proliferation (grow state), invasion (go state) and resistance to therapies. Thus, autophagy may function as a mechanism of tumor cell survival and progression in a hostile microenvironment. The intrinsic signals activating autophagy in GBM consist of specific gene expression levels alterations (like in the cMET gene), mutations (like the mutation in the EGFR gene that gives rise to the active mutant EGFRvIII) and/or specific signaling pathways perturbations (such as in the Wnt pathway). The extrinsic signals associated with autophagy activation in GBM cells are: 1) the perinecrotic niches (PNN), composed by highly proliferative GBM cells and where the autophagy activation may function as a cellular adaptive response to hypoxia; 2) the perivascular niche (PVN), where the vascular endothelial cells can interact with the GBM ones, inducing autophagy and a stemness phenotype of those tumor cells; 3) the brain extracellular matrix (bECM), whose components may regulate autophagy; and 4) the anticancer therapies, which can activate autophagy as a cytoprotective mechanism.

Due to these positive results in the early clinical trials, it is essential to invest in studies to evaluate CQ effects on GBM patients' survival using larger cohorts. Moreover, it is also necessary to determine HCQ efficacy and tolerated doses and invest in discovering new drugs with similar action mechanisms.

FUTURE DIRECTIONS: CROSSING THE VALLEY OF DEATH

The discrepancy between pro- and anti-tumor functions of autophagy, modulating GBM cell plasticity or alternative mechanism of cell death, emphasizes a question that has emerged as critical in translational science: how wide gap exists between basic and clinical biomedical data? The establishment of interdisciplinary research institutes stimulating collaborations between clinicians, physician-scientists, and basic biologists are critical to bring these areas together, but the importance of the critical interpretive reviews of literature data is also fundamental.

By examining carefully the literature we realize that explanations for the controversies of whether the autophagy pathway promotes survival or death are still elusive. Sometimes the balance between autophagic-dependent pro-survival or pro-death signals depends greatly on the quantitative relationship between them: over to moderate level of autophagy activation is cytoprotective, whereas high levels of autophagy are cytotoxic. Sometimes there are even conflicting reports with the same drug treatment in the same experimental model. Pre-clinical and clinical data indicate that autophagy is an emblematic example of a rescue pathway that contributes profoundly to a pro-tumoral adaptive response. On the other hand, high levels of activation lead to cytotoxic autophagy, which seems to be exclusively induced by excessive and homogeneous stress signals from *in vitro* cell-based studies.

From a standpoint of understanding the real GBM disease, the spatial and temporal heterogeneity of the external and internal stimuli must be considered. Steep gradients in pO2, pH, nutrient availability and drug perfusions ranging from pathological/therapeutic conditions to those found in normal tissues, added to the high levels of genetic intratumoral heterogeneity, are hallmark features of GBM. Thus, it implies that the levels of autophagy activation may also show extensive spatial heterogeneity in subpopulations from the same tumor determining divergent cell fates.

Despite the ability of many compounds, like CQ and HCQ, to inhibit autophagy and demonstrated good efficacy in preclinical studies, clinical trials for GBM continue showing no significant survival or clinical benefit, due to sparse anti-glioma activity or severe side effects. Thus, the last frontier to test the therapeutic potential of autophagy pathway in GBM awaits the development of compounds that can achieve more consistent inhibition.

Finally, only combinatorial therapy targeting autophagy with cytotoxic drugs in the adjuvant setting for GBM patients, associated with the development of less toxic and higher specific autophagy inhibitors, may inhibit adaptive response and enhance the sensibility of glioma cells to conventional therapies. In the context of an incurable human disease, pharmacological inhibition of autophagy would represent a promisor therapeutic target for radio- and chemosensitization of GBM cells.

AUTHOR CONTRIBUTIONS

EHFJ, MB, and LTI wrote the review and designed the figures. FBF and AAC critically revised the final version and ETC edited and supervised the whole work. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from FAPESP (16/07463-4, 16/06857-9), Ludwig Institute for Cancer Research and Hospital Sírio-Libanês.

REFERENCES

1. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: A Summary. *Acta Neuropathol* (2016) 131(6):803–20. doi: 10.1007/s00401-016-1545-1
2. Furnari FB, Fenton T, Bachoo RM, Mukasa A, Stommel JM, Stegh A, et al. Malignant Astrocytic Glioma: Genetics, Biology, and Paths to Treatment. *Genes Dev* (2007) 21(21):2683–710. doi: 10.1101/gad.1596707
3. Lowe S, Bhat KP, Olar A. Current Clinical Management of Patients With Glioblastoma. *Cancer Rep (Hoboken)* (2019) 2(6):e1216. doi: 10.1002/cnr2.1216
4. Tan AC, Ashley DM, López GY, Malinzak M, Friedman HS, Khasraw M. Management of Glioblastoma: State of the Art and Future Directions. *CA Cancer J Clin* (2020) 70(4):299–312. doi: 10.3322/caac.21613
5. Shen S, Clairambault J. Cell Plasticity in Cancer Cell Populations. *F1000Res* (2020) 9:F1000. doi: 10.12688/f1000research.24803.1
6. Ostrom QT, Cioffi G, Gittleman H, Patil N, Waite K, Kruchko C, et al. CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2012-2016. *Neuro Oncol* (2019) 21(Suppl 5):v1–v100. doi: 10.1093/neuonc/noz150
7. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy Plus Concomitant and Adjuvant Temozolomide for Glioblastoma. *N Engl J Med* (2005) 352(10):987–96. doi: 10.1056/NEJMoa043330
8. Andrade-Tomaz M, de Souza I, Rocha CRR, Gomes LR. The Role of Chaperone-Mediated Autophagy in Cell Cycle Control and Its Implications in Cancer. *Cells* (2020) 9(9):2140. doi: 10.3390/cells9092140
9. Auzmendi-Iriarte J, Matheu A. Impact of Chaperone-Mediated Autophagy in Brain Aging: Neurodegenerative Diseases and Glioblastoma. *Front Aging Neurosci* (2020) 12:630743. doi: 10.3389/fnagi.2020.630743
10. Coelho BP, Fernandes CFL, Boccacino JM, Souza MCDS, Melo-Escobar MI, Alves RN, et al. Multifaceted WNT Signaling at the Crossroads Between Epithelial-Mesenchymal Transition and Autophagy in Glioblastoma. *Front Oncol* (2020) 10:597743. doi: 10.3389/fonc.2020.597743
11. Sung K, Jimenez-Sanchez M. Autophagy in Astrocytes and its Implications in Neurodegeneration. *J Mol Biol* (2020) 432(8):2605–21. doi: 10.1016/j.jmb.2019.12.041

12. Teckman JH, Perlmutter DH. Retention of Mutant Alpha(1)-Antitrypsin Z in Endoplasmic Reticulum is Associated With an Autophagic Response. *Am J Physiol Gastrointest Liver Physiol* (2000) 279(5):G961–74. doi: 10.1152/ajpgi.2000.279.5.G961
13. Elmore SP, Qian T, Grissom SF, Lemasters JJ. The Mitochondrial Permeability Transition Initiates Autophagy in Rat Hepatocytes. *FASEB J* (2001) 15(12):2286–7. doi: 10.1096/fj.01-0206fje
14. Xue L, Fletcher GC, Tolkovsky AM. Mitochondria are Selectively Eliminated From Eukaryotic Cells After Blockade of Caspases During Apoptosis. *Curr Biol* (2001) 11(5):361–5. doi: 10.1016/s0960-9822(01)00100-2
15. Kondo Y, Kanzawa T, Sawaya R, Kondo S. The Role of Autophagy in Cancer Development and Response to Therapy. *Nat Rev Cancer* (2005) 5(9):726–34. doi: 10.1038/nrc1692
16. Duan S. Silencing the Autophagy-Specific Gene Beclin-1 Contributes to Attenuated Hypoxia-Induced Vasculogenic Mimicry Formation in Glioma. *Cancer Biomark* (2018) 21(3):565–74. doi: 10.3233/cbm-170444
17. Giatromanolaki A, Sivridis E, Mitrakas A, Kalamida D, Zois CE, Haider S, et al. Autophagy and Lysosomal Related Protein Expression Patterns in Human Glioblastoma. *Cancer Biol Ther* (2014) 15(11):1468–78. doi: 10.4161/15384047.2014.955719
18. Sivridis E, Koukourakis MI, Zois CE, Ledaki I, Ferguson DJ, Harris AL, et al. LC3A-Positive Light Microscopy Detected Patterns of Autophagy and Prognosis in Operable Breast Carcinomas. *Am J Pathol* (2010) 176 (5):2477–89. doi: 10.2353/ajpath.2010.090049
19. Koukourakis MI, Kalamida D, Mitrakas A, Pouliliou S, Kalamida S, Sivridis E, et al. Intensified Autophagy Compromises the Efficacy of Radiotherapy Against Prostate Cancer. *Biochem Biophys Res Commun* (2015) 461(2):268–74. doi: 10.1016/j.bbrc.2015.04.014
20. Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. Role of Autophagy in Temozolomide-Induced Cytotoxicity for Malignant Glioma Cells. *Cell Death Differ* (2004) 11(4):448–57. doi: 10.1038/sj.cdd.4401359
21. Sui X, Chen R, Wang Z, Huang Z, Kong N, Zhang M, et al. Autophagy and Chemotherapy Resistance: A Promising Therapeutic Target for Cancer Treatment. *Cell Death Dis* (2013) 4:e838. doi: 10.1038/cddis.2013.350
22. Natsumeda M, Aoki H, Miyahara H, Yajima N, Uzuka T, Toyoshima Y, et al. Induction of Autophagy in Temozolomide Treated Malignant Gliomas. *Neuropathology* (2011) 31(5):486–93. doi: 10.1111/j.1440-1789.2010.01197.x
23. Min Z, Ting Y, Mingtao G, Xiaofei T, Dong Y, Chenguang Z, et al. Monitoring Autophagic Flux Using p62/SQSTM1 Based Luciferase Reporters in Glioma Cells. *Exp Cell Res* (2018) 363(1):84–94. doi: 10.1016/j.yexcr.2017.12.027
24. Ito H, Daido S, Kanzawa T, Kondo S, Kondo Y. Radiation-Induced Autophagy is Associated With LC3 and its Inhibition Sensitizes Malignant Glioma Cells. *Int J Oncol* (2005) 26(5):1401–10. doi: 10.3892/ijo.26.5.1401
25. Yuan X, Du J, Hua S, Zhang H, Gu C, Wang J, et al. Suppression of Autophagy Augments the Radiosensitizing Effects of STAT3 Inhibition on Human Glioma Cells. *Exp Cell Res* (2015) 330(2):267–76. doi: 10.1016/j.yexcr.2014.09.006
26. Kroemer G, Levine B. Autophagic Cell Death: The Story of a Misnomer. *Nat Rev Mol Cell Biol* (2008) 9(12):1004–10. doi: 10.1038/nrm2529
27. Galluzzi L, Vitale I, Aaronson SA, Abrams JM, Adam D, Agostinis P, et al. Molecular Mechanisms of Cell Death: Recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death Differ* (2018) 25 (3):486–541. doi: 10.1038/s41418-017-0012-4
28. Noch E, Khalili K. Molecular Mechanisms of Necrosis in Glioblastoma: The Role of Glutamate Excitotoxicity. *Cancer Biol Ther* (2009) 8(19):1791–7. doi: 10.4161/cbt.8.19.9762
29. Barker FG, Davis RL, Chang SM, Prados MD. Necrosis as a Prognostic Factor in Glioblastoma Multiforme. *Cancer* (1996) 77(6):1161–6. doi: 10.1002/(sici)1097-0142(19960315)77:6<1161::aid-cncr24>3.0.co;2-z
30. Rong Y, Durden DL, Van Meir EG, Brat DJ. 'Pseudopalisading' Necrosis in Glioblastoma: A Familiar Morphologic Feature That Links Vascular Pathology, Hypoxia, and Angiogenesis. *J Neuropathol Exp Neurol* (2006) 65(6):529–39. doi: 10.1097/00005072-200606000-00001
31. Monteiro AR, Hill R, Pilkington GJ, Madureira PA. The Role of Hypoxia in Glioblastoma Invasion. *Cells* (2017) 6(4):45. doi: 10.3390/cells6040045
32. Bronisz A, Salińska E, Chiocca EA, Godlewski J. Hypoxic Roadmap of Glioblastoma-Learning About Directions and Distances in the Brain Tumor Environment. *Cancers (Basel)* (2020) 12(5):1213. doi: 10.3390/cancers12051213
33. Jawhari S, Ratinaud MH, Verdier M. Glioblastoma, Hypoxia and Autophagy: A Survival-Prone 'Ménage-À-Trois'. *Cell Death Dis* (2016) 7 (10):e2434. doi: 10.1038/cddis.2016.318
34. Semenza GL. Targeting HIF-1 for Cancer Therapy. *Nat Rev Cancer* (2003) 3 (10):721–32. doi: 10.1038/nrc1187
35. Li Z, Bao S, Wu Q, Wang H, Eyler C, Sathornsumetee S, et al. Hypoxia-Inducible Factors Regulate Tumorigenic Capacity of Glioma Stem Cells. *Cancer Cell* (2009) 15(6):501–13. doi: 10.1016/j.ccr.2009.03.018
36. Wolf A, Agnihotri S, Guha A. Targeting Metabolic Remodeling in Glioblastoma Multiforme. *Oncotarget* (2010) 1(7):552–62. doi: 10.18632/oncotarget.190
37. Jensen RL, Mumert ML, Gillespie DL, Kinney AY, Schabel MC, Salzman KL. Preoperative Dynamic Contrast-Enhanced MRI Correlates With Molecular Markers of Hypoxia and Vascularity in Specific Areas of Intratumoral Microenvironment and is Predictive of Patient Outcome. *Neuro Oncol* (2014) 16(2):280–91. doi: 10.1093/neuonc/not148
38. Teicher BA. Hypoxia and Drug Resistance. *Cancer Metastasis Rev* (1994) 13 (2):139–68. doi: 10.1007/bf00689633
39. Harris AL. Hypoxia–a Key Regulatory Factor in Tumour Growth. *Nat Rev Cancer* (2002) 2(1):38–47. doi: 10.1038/nrc704
40. Persano L, Pistollato F, Rampazzo E, Della Puppa A, Abbadi S, Frasson C, et al. BMP2 Sensitizes Glioblastoma Stem-Like Cells to Temozolomide by Affecting HIF-1α Stability and MGMT Expression. *Cell Death Dis* (2012) 3: e412. doi: 10.1038/cddis.2012.153
41. Ge X, Pan MH, Wang L, Li W, Jiang C, He J, et al. Hypoxia-Mediated Mitochondria Apoptosis Inhibition Induces Temozolomide Treatment Resistance Through miR-26a/Bad/Bax Axis. *Cell Death Dis* (2018) 9 (11):1128. doi: 10.1038/s41419-018-1176-7
42. Pope WB, Xia Q, Paton VE, Das A, Hambleton J, Kim HJ, et al. Patterns of Progression in Patients With Recurrent Glioblastoma Treated With Bevacizumab. *Neurology* (2011) 76(5):432–7. doi: 10.1212/WNL.0b013e31820a0a8a
43. Swietach P, Vaughan-Jones RD, Harris AL. Regulation of Tumor Ph and the Role of Carbonic Anhydrase 9. *Cancer Metastasis Rev* (2007) 26(2):299–310. doi: 10.1007/s10555-007-9064-0
44. Kundu S, Xiong A, Spyrou A, Wicher G, Marinescu VD, Edqvist PD, et al. Heparanase Promotes Glioma Progression and Is Inversely Correlated With Patient Survival. *Mol Cancer Res* (2016) 14(12):1243–53. doi: 10.1158/1541-7786.Mcr-16-0223
45. Tran VM, Wade A, McKinney A, Chen K, Lindberg OR, Engler JR, et al. Heparan Sulfate Glycosaminoglycans in Glioblastoma Promote Tumor Invasion. *Mol Cancer Res* (2017) 15(11):1623–33. doi: 10.1158/1541-7786.Mcr-17-0352
46. Sanderson RD, Elkin M, Rapraeger AC, Ilan N, Vlodavsky I. Heparanase Regulation of Cancer, Autophagy and Inflammation: New Mechanisms and Targets for Therapy. *FEBS J* (2017) 284(1):42–55. doi: 10.1111/febs.13932
47. Shteingauz A, Boyango I, Naroditsky I, Hammond E, Gruber M, Doweck I, et al. Heparanase Enhances Tumor Growth and Chemoresistance by Promoting Autophagy. *Cancer Res* (2015) 75(18):3946–57. doi: 10.1158/0008-5472.Can-15-0037
48. Koh SP, Wickremesekera AC, Brasch HD, Marsh R, Tan ST, Itinteang T. Expression of Cathepsins B, D, and G in Isocitrate Dehydrogenase-Wildtype Glioblastoma. *Front Surg* (2017) 4:28. doi: 10.3389/fsurg.2017.00028
49. Yadati T, Houben T, Bitorina A, Shiri-Sverdlov R. Physiological Function and Role in Disease Management. *Cells* (2020) 9(7):1679. doi: 10.3390/cells9071679
50. Bischof J, Westhoff MA, Wagner JE, Halatsch ME, Trentmann S, Knippschild U, et al. Cancer Stem Cells: The Potential Role of Autophagy, Proteolysis, and Cathepsins in Glioblastoma Stem Cells. *Tumour Biol* (2017) 39(3):1010428317692227. doi: 10.1177/1010428317692227
51. Formolo CA, Williams R, Gordish-Dressman H, MacDonald TJ, Lee NH, Hathout Y. Secretome Signature of Invasive Glioblastoma Multiforme. *J Proteome Res* (2011) 10(7):3149–59. doi: 10.1021/pr200210w
52. Gole B, Huszthy PC, Popović M, Jeruc J, Ardebili YS, Bjerkvig R, et al. The Regulation of Cysteine Cathepsins and Cystatins in Human Gliomas. *Int J Cancer* (2012) 131(8):1779–89. doi: 10.1002/ijc.27453

53. Pišlar A, Jewett A, Kos J. Cysteine Cathepsins: Their Biological and Molecular Significance in Cancer Stem Cells. *Semin Cancer Biol* (2018) 53:168–77. doi: 10.1016/j.semcancer.2018.07.010
54. Sivaparvathi M, Sawaya R, Chintala SK, Go Y, Gokaslan ZL, Rao JS. Expression of Cathepsin D During the Progression of Human Gliomas. *Neurosci Lett* (1996) 208(3):171–4. doi: 10.1016/0304-3940(96)12584-2
55. Zheng W, Chen Q, Wang C, Yao D, Zhu L, Pan Y, et al. Inhibition of Cathepsin D (CTSD) Enhances Radiosensitivity of Glioblastoma Cells by Attenuating Autophagy. *Mol Carcinog* (2020) 59(6):651–60. doi: 10.1002/mc.23194
56. Lamark T, Kirkin V, Dikic I, Johansen T. NBR1 and p62 as Cargo Receptors for Selective Autophagy of Ubiquitinated Targets. *Cell Cycle* (2009) 8 (13):1986–90. doi: 10.4161/cc.8.13.8892
57. Sivaparvathi M, Yamamoto M, Nicolson GL, Gokaslan ZL, Fuller GN, Liotta LA, et al. Expression and Immunohistochemical Localization of Cathepsin L During the Progression of Human Gliomas. *Clin Exp Metastasis* (1996) 14 (1):27–34. doi: 10.1007/bf00157683
58. Levicar N, Dewey RA, Daley E, Bates TE, Davies D, Kos J, et al. Selective Suppression of Cathepsin L by Antisense cDNA Impairs Human Brain Tumor Cell Invasion In Vitro and Promotes Apoptosis. *Cancer Gene Ther* (2003) 10(2):141–51. doi: 10.1038/sj.cgt.7700546
59. Xiong Y, Ji W, Fei Y, Zhao Y, Wang L, Wang W, et al. Cathepsin L is Involved in X-ray-induced Invasion and Migration of Human Glioma U251 Cells. *Cell Signal* (2017) 29:181–91. doi: 10.1016/j.cellsig.2016.10.012
60. Zhang X, Xu R, Zhang C, Xu Y, Han M, Huang B, et al. Trifluoperazine, a Novel Autophagy Inhibitor, Increases Radiosensitivity in Glioblastoma by Impairing Homologous Recombination. *J Exp Clin Cancer Res* (2017) 36 (1):118. doi: 10.1186/s13046-017-0588-z
61. Wu J, Lei Z, Yu J. Hypoxia Induces Autophagy in Human Vascular Endothelial Cells in a Hypoxia-Inducible Factor 1–Dependent Manner. *Mol Med Rep* (2015) 11(4):2677–82. doi: 10.3892/mmr.2014.3093
62. Denton D, Nicolson S, Kumar S. Cell Death by Autophagy: Facts and Apparent Artefacts. *Cell Death Differ* (2012) 19(1):87–95. doi: 10.1038/cdd.2011.146
63. Park SY, Sun EG, Lee Y, Kim MS, Kim JH, Kim WJ, et al. Autophagy Induction Plays a Protective Role Against Hypoxic Stress in Human Dental Pulp Cells. *J Cell Biochem* (2018) 119(2):1992–2002. doi: 10.1002/jcb.26360
64. Lu N, Li X, Tan R, An J, Cai Z, Hu X, et al. HIF-1α/Beclin1-Mediated Autophagy is Involved in Neuroprotection Induced by Hypoxic Preconditioning. *J Mol Neurosci* (2018) 66(2):238–50. doi: 10.1007/s12031-018-1162-7
65. Menon MB, Dhamija S. Beclin 1 Phosphorylation - at the Center of Autophagy Regulation. *Front Cell Dev Biol* (2018) 6:137. doi: 10.3389/fcell.2018.00137
66. Qian X, Li X, Cai Q, Zhang C, Yu Q, Jiang Y, et al. Phosphoglycerate Kinase 1 Phosphorylates Beclin1 to Induce Autophagy. *Mol Cell* (2017) 65(5):917–31. doi: 10.1016/j.molcel.2017.01.027
67. Rolhion C, Penault-Llorca F, Kémény JL, Lemaire JJ, Jullien C, Labit-Bouvier C, et al. Interleukin-6 Overexpression as a Marker of Malignancy in Human Gliomas. *J Neurosurg* (2001) 94(1):97–101. doi: 10.3171/jns.2001.94.1.0097
68. Joseph JV, Conroy S, Pavlov K, Sontakke P, Tomar T, Eggens-Meijer E, et al. Hypoxia Enhances Migration and Invasion in Glioblastoma by Promoting a Mesenchymal Shift Mediated by the HIF1α-ZEB1 Axis. *Cancer Lett* (2015) 359(1):107–16. doi: 10.1016/j.canlet.2015.01.010
69. Xue H, Yuan G, Guo X, Liu Q, Zhang J, Gao X, et al. A Novel Tumor-Promoting Mechanism of IL6 and the Therapeutic Efficacy of Tocilizumab: Hypoxia-induced IL6 is a Potent Autophagy Initiator in Glioblastoma Via the p-STAT3-MIR155-3p-CREBRF Pathway. *Autophagy* (2016) 12(7):1129–52. doi: 10.1080/15548627.2016.1178446
70. Xue H, Zhang J, Guo X, Wang J, Li J, Gao X, et al. CREBRF is a Potent Tumor Suppressor of Glioblastoma by Blocking Hypoxia-Induced Autophagy Via the CREB3/ATG5 Pathway. *Int J Oncol* (2016) 49(2):519–28. doi: 10.3892/ijo.2016.3576
71. Liu Q, Li G, Li R, Shen J, He Q, Deng L, et al. IL-6 Promotion of Glioblastoma Cell Invasion and Angiogenesis in U251 and T98G Cell Lines. *J Neurooncol* (2010) 100(2):165–76. doi: 10.1007/s11060-010-0158-0
72. Azad MB, Chen Y, Henson ES, Cizeau J, McMillan-Ward E, Israels SJ, et al. Hypoxia Induces Autophagic Cell Death in Apoptosis-Competent Cells Through a Mechanism Involving BNIP3. *Autophagy* (2008) 4(2):195–204. doi: 10.4161/auto.5278
73. Bellot G, Garcia-Medina R, Gounon P, Chiche J, Roux D, Pouysségur J, et al. Hypoxia-Induced Autophagy is Mediated Through Hypoxia-Inducible Factor Induction of BNIP3 and BNIP3L Via Their BH3 Domains. *Mol Cell Biol* (2009) 29(10):2570–81. doi: 10.1128/mcb.00166-09
74. López-Camarillo C, Ruiz-García E, Starling N, Marchat LA. Editorial: Neovascularization, Angiogenesis and Vasculogenic Mimicry in Cancer. *Front Oncol* (2020) 10:1140. doi: 10.3389/fonc.2020.01140
75. Ulrich TA, de Juan Pardo EM, Kumar S. The Mechanical Rigidity of the Extracellular Matrix Regulates the Structure, Motility, and Proliferation of Glioma Cells. *Cancer Res* (2009) 69(10):4167–74. doi: 10.1158/0008-5472.CAN-08-4859
76. Giese A, Kluwe L, Laube B, Meissner H, Berens ME, Westphal M. Migration of Human Glioma Cells on Myelin. *Neurosurgery* (1996) 38(4):755–64. doi: 10.1227/00006123-199604000-00026
77. Bellail AC, Hunter SB, Brat DJ, Tan C, Van Meir EG. Microregional Extracellular Matrix Heterogeneity in Brain Modulates Glioma Cell Invasion. *Int J Biochem Cell Biol* (2004) 36(6):1046–69. doi: 10.1016/j.biocel.2004.01.013
78. Kreisl TN, Kim L, Moore K, Duic P, Royce C, Stroud I, et al. Phase II Trial of Single-Agent Bevacizumab Followed by Bevacizumab Plus Irinotecan at Tumor Progression in Recurrent Glioblastoma. *J Clin Oncol* (2009) 27 (5):740–5. doi: 10.1200/jco.2008.16.3055
79. Wu HB, Yang S, Weng HY, Chen Q, Zhao XL, Fu WJ, et al. Autophagy-Induced KDR/VEGFR-2 Activation Promotes the Formation of Vasculogenic Mimicry by Glioma Stem Cells. *Autophagy* (2017) 13 (9):1528–42. doi: 10.1080/15548627.2017.1336277
80. Angara K, Rashid MH, Shankar A, Ara R, Iskander A, Borin TF, et al. Vascular Mimicry in Glioblastoma Following Anti-Angiogenic and Anti-20-HETE Therapies. *Histol Histopathol* (2017) 32(9):917–28. doi: 10.14670/HH-11-856
81. Angara K, Borin TF, Rashid MH, Lebedyeva I, Ara R, Lin PC, et al. CXCR2-Expressing Tumor Cells Drive Vascular Mimicry in Antiangiogenic Therapy-Resistant Glioblastoma. *Neoplasia* (2018) 20(10):1070–82. doi: 10.1016/j.neo.2018.08.011
82. West AJ, Tsui V, Stylli SS, Nguyen HPT, Morokoff AP, Kaye AH, et al. The Role of Interleukin-6-STAT3 Signalling in Glioblastoma. *Oncol Lett* (2018) 16(4):4095–104. doi: 10.3892/ol.2018.9227
83. Gubbiotti MA, Iozzo RV. Proteoglycans Regulate Autophagy Via Outside-in Signaling: An Emerging New Concept. *Matrix Biol* (2015) 48:6–13. doi: 10.1016/j.matbio.2015.10.002
84. Neill T, Buraschi S, Kapoor A, Iozzo RV. Proteoglycan-Driven Autophagy: A Nutrient-Independent Mechanism to Control Intracellular Catabolism. *J Histochem Cytochem* (2020) 68(11):733–46. doi: 10.1369/0022155420937370
85. Petterson SA, Dahlrot RH, Hermansen SK, K A Munthe S, Gundesen MT, Wohlleben H, et al. High Levels of c-Met is Associated With Poor Prognosis in Glioblastoma. *J Neurooncol* (2015) 122(3):517–27. doi: 10.1007/s11060-015-1723-3
86. Olmez OF, Cubukcu E, Evrensel T, Kurt M, Avci N, Tolunay S, et al. The Immunohistochemical Expression of c-Met is an Independent Predictor of Survival in Patients With Glioblastoma Multiforme. *Clin Transl Oncol* (2014) 16(2):173–7. doi: 10.1007/s12094-013-1059-4
87. Liu W, Fu Y, Xu S, Ding F, Zhao G, Zhang K, et al. c-Met Expression is Associated With Time to Recurrence in Patients With Glioblastoma Multiforme. *J Clin Neurosci* (2011) 18(1):119–21. doi: 10.1016/j.jocn.2010.05.010
88. Tsidulko AY, Kazanskaya GM, Volkov AM, Suhovskih AV, Kiselev RS, Kobozev VV, et al. Chondroitin Sulfate Content and Decorin Expression in Glioblastoma are Associated With Proliferative Activity of Glioma Cells and Disease Prognosis. *Cell Tissue Res* (2020) 379(1):147–55. doi: 10.1007/s00441-019-03127-2
89. Buraschi S, Neill T, Goyal A, Poluzzi C, Smythies J, Owens RT, et al. Decorin Causes Autophagy in Endothelial Cells Via Peg3. *Proc Natl Acad Sci USA* (2013) 110(28):E2582–91. doi: 10.1073/pnas.1305732110
90. Buraschi S, Neill T, Iozzo RV. Decorin is a Devouring Proteoglycan: Remodeling of Intracellular Catabolism Via Autophagy and Mitophagy. *Matrix Biol* (2019) 75-76:260–70. doi: 10.1016/j.matbio.2017.10.005

91. Farace C, Oliver JA, Melguizo C, Alvarez P, Bandiera P, Rama AR, et al. Microenvironmental Modulation of Decorin and Lumican in Temozolomide-Resistant Glioblastoma and Neuroblastoma Cancer Stem-Like Cells. *PloS One* (2015) 10(7):e0134111. doi: 10.1371/journal.pone.0134111
92. Bi X, Pohl NM, Qian Z, Yang GR, Gou Y, Guzman G, et al. Decorin-Mediated Inhibition of Colorectal Cancer Growth and Migration is Associated With E-Cadherin In Vitro and in Mice. *Carcinogenesis* (2012) 33(2):326–30. doi: 10.1093/carcin/bgr293
93. Stock C, Jungmann O, Seidler DG. Decorin and Chondroitin-6 Sulfate Inhibit B16V Melanoma Cell Migration and Invasion by Cellular Acidification. *J Cell Physiol* (2011) 226(10):2641–50. doi: 10.1002/jcp.22612
94. Yao T, Zhang CG, Gong MT, Zhang M, Wang L, Ding W. Decorin-Mediated Inhibition of the Migration of U87MG Glioma Cells Involves Activation of Autophagy and Suppression of TGF-β Signaling. *FEBS Open Bio* (2016) 6 (7):707–19. doi: 10.1002/2211-5463.12076
95. Douglass S, Goyal A, Iozzo RV. The Role of Perlecan and Endorepellin in the Control of Tumor Angiogenesis and Endothelial Cell Autophagy. *Connect Tissue Res* (2015) 56(5):381–91. doi: 10.3109/03008207.2015.1045297
96. Neill T, Andreuzzi E, Wang ZX, Peiper SC, Mongiat M, Iozzo RV. Endorepellin Remodels the Endothelial Transcriptome Toward a Pro-Autophagic and Pro-Mitophagic Gene Signature. *J Biol Chem* (2018) 293 (31):12137–48. doi: 10.1074/jbc.RA118.002934
97. Calabrese C, Poppleton H, Kocak M, Hogg TL, Fuller C, Hamner B, et al. A Perivascular Niche for Brain Tumor Stem Cells. *Cancer Cell* (2007) 11 (1):69–82. doi: 10.1016/j.ccr.2006.11.020
98. Seano G. Targeting the Perivascular Niche in Brain Tumors. *Curr Opin Oncol* (2018) 30(1):54–60. doi: 10.1097/cco.0000000000000417
99. Cuddapah VA, Robel S, Watkins S, Sontheimer H. A Neurocentric Perspective on Glioma Invasion. *Nat Rev Neurosci* (2014) 15(7):455–65. doi: 10.1038/nrn3765
100. Montana V, Sontheimer H. Bradykinin Promotes the Chemotactic Invasion of Primary Brain Tumors. *J Neurosci* (2011) 31(13):4858–67. doi: 10.1523/jneurosci.3825-10.2011
101. Hossain A, Gumin J, Gao F, Figueroa J, Shinojima N, Takezaki T, et al. Mesenchymal Stem Cells Isolated From Human Gliomas Increase Proliferation and Maintain Stemness of Glioma Stem Cells Through the IL-6/gp130/STAT3 Pathway. *Stem Cells* (2015) 33(8):2400–15. doi: 10.1002/stem.2053
102. Charles N, Holland EC. The Perivascular Niche Microenvironment in Brain Tumor Progression. *Cell Cycle* (2010) 9(15):3012–21. doi: 10.4161/cc.9.15.12710
103. Diksin M, Smith SJ, Rahman R. The Molecular and Phenotypic Basis of the Glioma Invasive Perivascular Niche. *Int J Mol Sci* (2017) 18(11):2342. doi: 10.3390/ijms18112342
104. Schiffer D, Annovazzi L, Casalone C, Corona C, Mellai M. Glioblastoma: Microenvironment and Niche Concept. *Cancers (Basel)* (2018) 11(1):5. doi: 10.3390/cancers11010005
105. Molina ML, García-Bernal D, Martinez S, Valdor R. Autophagy in the Immunosuppressive Perivascular Microenvironment of Glioblastoma. *Cancers (Basel)* (2019) 12(1):102. doi: 10.3390/cancers12010102
106. Clarke AJ, Simon AK. Autophagy in the Renewal, Differentiation and Homeostasis of Immune Cells. *Nat Rev Immunol* (2019) 19(3):170–83. doi: 10.1038/s41577-018-0095-2
107. Valdor R, García-Bernal D, Riquelme D, Martinez CM, Moraleda JM, Cuervo AM, et al. Glioblastoma Ablates Pericytes Antitumor Immune Function Through Aberrant Up-Regulation of Chaperone-Mediated Autophagy. *Proc Natl Acad Sci USA* (2019) 116(41):20655–65. doi: 10.1073/pnas.1903542116
108. Nussenzweig SC, Verma S, Finkel T. The Role of Autophagy in Vascular Biology. *Circ Res* (2015) 116(3):480–8. doi: 10.1161/circresaha.116.303805
109. Ma Y, Wang P, Xue Y, Qu C, Zheng J, Liu X, et al. PVT1 Affects Growth of Glioma Microvascular Endothelial Cells by Negatively Regulating Mir-186. *Tumour Biol* (2017) 39(3):1010428317694326. doi: 10.1177/1010428317694326
110. Pietras A, Katz AM, Ekström EJ, Wee B, Halliday JJ, Pitter KL, et al. Osteopontin-CD44 Signaling in the Glioma Perivascular Niche Enhances Cancer Stem Cell Phenotypes and Promotes Aggressive Tumor Growth. *Cell Stem Cell* (2014) 14(3):357–69. doi: 10.1016/j.stem.2014.01.005
111. Moorman HR, Poschel D, Klement JD, Lu C, Redd PS, Liu K. Osteopontin: A Key Regulator of Tumor Progression and Immunomodulation. *Cancers (Basel)* (2020) 12(11):3379. doi: 10.3390/cancers12113379
112. Jan HJ, Lee CC, Shih YL, Hueng DY, Ma HI, Lai JH, et al. Osteopontin Regulates Human Glioma Cell Invasiveness and Tumor Growth in Mice. *Neuro Oncol* (2010) 12(1):58–70. doi: 10.1093/neuonc/nop013
113. Pradhan AK, Maji S, Das SK, Emdad L, Sarkar D, Fisher PB. Mda-9/Syntenin/SDCBP: New Insights Into a Unique Multifunctional Scaffold Protein. *Cancer Metastasis Rev* (2020) 39(3):769–81. doi: 10.1007/s10555-020-09886-7
114. Kegelman TP, Das SK, Hu B, Bacolod MD, Fuller CE, Menezes ME, et al. MDA-9/syntenin is a Key Regulator of Glioma Pathogenesis. *Neuro Oncol* (2014) 16(1):50–61. doi: 10.1093/neuonc/not157
115. Talukdar S, Das SK, Pradhan AK, Emdad L, Shen XN, Windle JJ, et al. Novel Function of MDA-9/Syntenin (SDCBP) as a Regulator of Survival and Stemness in Glioma Stem Cells. *Oncotarget* (2016) 7(34):54102–19. doi: 10.18632/oncotarget.10851
116. Talukdar S, Pradhan AK, Bhoopathi P, Shen XN, August LA, Windle JJ, et al. MDA-9/Syntenin Regulates Protective Autophagy in Anoikis-Resistant Glioma Stem Cells. *Proc Natl Acad Sci USA* (2018) 115(22):5768–73. doi: 10.1073/pnas.1721650115
117. Fung C, Lock R, Gao S, Salas E, Debnath J. Induction of Autophagy During Extracellular Matrix Detachment Promotes Cell Survival. *Mol Biol Cell* (2008) 19(3):797–806. doi: 10.1091/mbc.e07-10-1092
118. Chen C, Kapoor A, Iozzo RV. Methods for Monitoring Matrix-Induced Autophagy. *Methods Mol Biol* (2019) 1952:157–91. doi: 10.1007/978-1-4939-9133-4_14
119. Bacolod MD, Das SK, Sokhi UK, Bradley S, Fenstermacher DA, Pellecchia M, et al. Examination of Epigenetic and Other Molecular Factors Associated With Mda-9/Syntenin Dysregulation in Cancer Through Integrated Analyses of Public Genomic Datasets. *Adv Cancer Res* (2015) 127:49–121. doi: 10.1016/bs.acr.2015.04.006
120. Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, et al. Effects of Radiotherapy With Concomitant and Adjuvant Temozolomide Versus Radiotherapy Alone on Survival in Glioblastoma in a Randomised Phase III Study: 5-Year Analysis of the EORTC-NCIC Trial. *Lancet Oncol* (2009) 10(5):459–66. doi: 10.1016/S1470-2045(09)70025-7
121. Gilbert MR, Dignam JJ, Armstrong TS, Wefel JS, Blumenthal DT, Vogelbaum MA, et al. A Randomized Trial of Bevacizumab for Newly Diagnosed Glioblastoma. *N Engl J Med* (2014) 370(8):699–708. doi: 10.1056/NEJMoa1308573
122. Knizhnik AV, Roos WP, Nikolova T, Quiros S, Tomaszowski KH, Christmann M, et al. Survival and Death Strategies in Glioma Cells: Autophagy, Senescence and Apoptosis Triggered by a Single Type of Temozolomide-Induced DNA Damage. *PloS One* (2013) 8(1):e55665. doi: 10.1371/journal.pone.0055665
123. Golden EB, Cho HY, Jahanian A, Hofman FM, Louie SG, Schönthal AH, et al. Chloroquine Enhances Temozolomide Cytotoxicity in Malignant Gliomas by Blocking Autophagy. *Neurosurg Focus* (2014) 37(6):E12. doi: 10.3171/2014.9.Focus14504
124. Liu X, Sun K, Wang H, Dai Y. Inhibition of Autophagy by Chloroquine Enhances the Antitumor Efficacy of Sorafenib in Glioblastoma. *Cell Mol Neurobiol* (2016) 36(7):1197–208. doi: 10.1007/s10571-015-0318-z
125. Yao KC, Komata T, Kondo Y, Kanzawa T, Kondo S, Germano IM. Molecular Response of Human Glioblastoma Multiforme Cells to Ionizing Radiation: Cell Cycle Arrest, Modulation of the Expression of Cyclin-Dependent Kinase Inhibitors, and Autophagy. *J Neurosurg* (2003) 98(2):378–84. doi: 10.3171/jns.2003.98.2.0378
126. Ye H, Chen M, Cao F, Huang H, Zhan R, Zheng X. Chloroquine, an Autophagy Inhibitor, Potentiates the Radiosensitivity of Glioma Initiating Cells by Inhibiting Autophagy and Activating Apoptosis. *BMC Neurol* (2016) 16(1):178. doi: 10.1186/s12883-016-0700-6
127. Daido S, Yamamoto A, Fujiwara K, Sawaya R, Kondo S, Kondo Y. Inhibition of the DNA-dependent Protein Kinase Catalytic Subunit Radiosensitizes Malignant Glioma Cells by Inducing Autophagy. *Cancer Res* (2005) 65 (10):4368–75. doi: 10.1158/0008-5472.Can-04-4202
128. Huang H, Song J, Liu Z, Pan L, Xu G. Autophagy Activation Promotes Bevacizumab Resistance in Glioblastoma by Suppressing Akt/mTOR

Signaling Pathway. *Oncol Lett* (2018) 15(2):1487–94. doi: 10.3892/ol.2017.7446
129. Liang J, Piao Y, Henry V, Tiao N, de Groot JF. Interferon-Regulatory Factor-1 (IRF1) Regulates Bevacizumab Induced Autophagy. *Oncotarget* (2015) 6 (31):31479–92. doi: 10.18632/oncotarget.5491
130. Müller-Greven G, Carlin CR, Burgett ME, Ahluwalia MS, Lauko A, Nowacki AS, et al. Macropinocytosis of Bevacizumab by Glioblastoma Cells in the Perivascular Niche Affects Their Survival. *Clin Cancer Res* (2017) 23 (22):7059–71. doi: 10.1158/1078-0432.Ccr-17-0249
131. Hu YL, DeLay M, Jahangiri A, Molinaro AM, Rose SD, Carbonell WS, et al. Hypoxia-Induced Autophagy Promotes Tumor Cell Survival and Adaptation to Antiangiogenic Treatment in Glioblastoma. *Cancer Res* (2012) 72 (7):1773–83. doi: 10.1158/0008-5472.Can-11-3831
132. Kang MR, Kim MS, Oh JE, Kim YR, Song SY, Kim SS, et al. Frameshift Mutations of Autophagy-Related Genes ATG2B, Atg5, ATG9B and ATG12 in Gastric and Colorectal Cancers With Microsatellite Instability. *J Pathol* (2009) 217(5):702–6. doi: 10.1002/path.2509
133. An CH, Kim MS, Yoo NJ, Park SW, Lee SH. Mutational and Expressional Analyses of ATG5, an Autophagy-Related Gene, in Gastrointestinal Cancers. *Pathol Res Pract* (2011) 207(7):433–7. doi: 10.1016/j.prp.2011.05.002
134. Lebovitz CB, Robertson AG, Goya R, Jones SJ, Morin RD, Marra MA, et al. Cross-Cancer Profiling of Molecular Alterations Within the Human Autophagy Interaction Network. *Autophagy* (2015) 11(9):1668–87. doi: 10.1080/15548627.2015.1067362
135. Amaravadi R, Kimmelman AC, White E. Recent Insights Into the Function of Autophagy in Cancer. *Genes Dev* (2016) 30(17):1913–30. doi: 10.1101/gad.287524.116
136. Wang QW, Liu HJ, Zhao Z, Zhang Y, Wang Z, Jiang T, et al. Prognostic Correlation of Autophagy-Related Gene Expression-Based Risk Signature in Patients With Glioblastoma. *Onco Targets Ther* (2020) 13:95–107. doi: 10.2147/ott.S238332
137. Wang Y, Zhao W, Xiao Z, Guan G, Liu X, Zhuang M. A Risk Signature With Four Autophagy-Related Genes for Predicting Survival of Glioblastoma Multiforme. *J Cell Mol Med* (2020) 24(7):3807–21. doi: 10.1111/jcmm.14938
138. Wang Z, Gao L, Guo X, Feng C, Lian W, Deng K, et al. Development and Validation of a Nomogram With an Autophagy-Related Gene Signature for Predicting Survival in Patients With Glioblastoma. *Aging (Albany NY)* (2019) 11(24):12246–69. doi: 10.18632/aging.102566
139. Tamrakar S, Yashiro M, Kawashima T, Uda T, Terakawa Y, Kuwae Y, et al. Clinicopathological Significance of Autophagy-related Proteins and its Association With Genetic Alterations in Gliomas. *Anticancer Res* (2019) 39(3):1233–42. doi: 10.21873/anticanres.13233
140. Zhao M, Xu H, Zhang B, Hong B, Yan W, Zhang J. Impact of Nuclear Factor Erythroid-Derived 2-Like 2 and p62/sequestosome Expression on Prognosis of Patients With Gliomas. *Hum Pathol* (2015) 46(6):843–9. doi: 10.1016/j.humpath.2015.02.009
141. Galavotti S, Bartesaghi S, Faccenda D, Shaked-Rabi M, Sanzone S, McEvoy A, et al. The Autophagy-Associated Factors DRAM1 and p62 Regulate Cell Migration and Invasion in Glioblastoma Stem Cells. *Oncogene* (2013) 32 (6):699–712. doi: 10.1038/onc.2012.111
142. Li H, Li J, Zhang G, Da Q, Chen L, Yu S, et al. HMGB1-Induced P62 Overexpression Promotes Snail-Mediated Epithelial-Mesenchymal Transition in Glioblastoma Cells Via the Degradation of GSK-3β. *Theranostics* (2019) 9(7):1909–22. doi: 10.7150/thno.30578
143. Wen ZP, Zeng WJ, Chen YH, Li H, Wang JY, Cheng Q, et al. Knockdown ATG4C Inhibits Gliomas Progression and Promotes Temozolomide Chemosensitivity by Suppressing Autophagic Flux. *J Exp Clin Cancer Res* (2019) 38(1):298. doi: 10.1186/s13046-019-1287-8
144. Brennan CW, Verhaak RG, McKenna A, Campos B, Noushmehr H, Salama SR, et al. The Somatic Genomic Landscape of Glioblastoma. *Cell* (2013) 155 (2):462–77. doi: 10.1016/j.cell.2013.09.034
145. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR Regulate Autophagy Through Direct Phosphorylation of Ulk1. *Nat Cell Biol* (2011) 13(2):132–41. doi: 10.1038/ncb2152
146. Wang RC, Wei Y, An Z, Zou Z, Xiao G, Bhagat G, et al. Akt-Mediated Regulation of Autophagy and Tumorigenesis Through Beclin 1 Phosphorylation. *Science* (2012) 338(6109):956–9. doi: 10.1126/science.1225967
147. Martelli AM, Evangelisti C, Follo MY, Ramazzotti G, Fini M, Giardino R, et al. Targeting the Phosphatidylinositol 3-Kinase/Akt/Mammalian Target of Rapamycin Signaling Network in Cancer Stem Cells. *Curr Med Chem* (2011) 18(18):2715–26. doi: 10.2174/092986711796011201
148. Eskilsson E, Røsland GV, Solecki G, Wang Q, Harter PN, Graziani G, et al. EGFR Heterogeneity and Implications for Therapeutic Intervention in Glioblastoma. *Neuro Oncol* (2018) 20(6):743–52. doi: 10.1093/neuonc/nox191
149. Li X, Wu C, Chen N, Gu H, Yen A, Cao L, et al. Pi3k/Akt/mTOR Signaling Pathway and Targeted Therapy for Glioblastoma. *Oncotarget* (2016) 7 (22):33440–50. doi: 10.18632/oncotarget.7961
150. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, et al. An Integrated Genomic Analysis of Human Glioblastoma Multiforme. *Science* (2008) 321(5897):1807–12. doi: 10.1126/science.1164382
151. Koul D. PTEN Signaling Pathways in Glioblastoma. *Cancer Biol Ther* (2008) 7(9):1321–5. doi: 10.4161/cbt.7.9.6954
152. Álvarez-Garcia V, Tawil Y, Wise HM, Leslie NR. Mechanisms of PTEN Loss in Cancer: It's All About Diversity. *Semin Cancer Biol* (2019) 59:66–79. doi: 10.1016/j.semcancer.2019.02.001
153. Koul D, Shen R, Kim YW, Kondo Y, Lu Y, Bankson J, et al. Cellular and In Vivo Activity of a Novel PI3K Inhibitor, PX-866, Against Human Glioblastoma. *Neuro Oncol* (2010) 12(6):559–69. doi: 10.1093/neuonc/nop058
154. Ferese R, Lenzi P, Fulceri F, Biagioni F, Fabrizi C, Gambardella S, et al. Quantitative Ultrastructural Morphometry and Gene Expression of Mtor-Related Mitochondriogenesis Within Glioblastoma Cells. *Int J Mol Sci* (2020) 21(13):4570. doi: 10.3390/ijms21134570
155. Zhang Y, Dube C, Gibert M, Cruickshanks N, Wang B, Coughlan M, et al. The P53 Pathway in Glioblastoma. *Cancers (Basel)* (2018) 10(9):297. doi: 10.3390/cancers10090297
156. Crighton D, Wilkinson S, O'Prey J, Syed N, Smith P, Harrison PR, et al. DRAM, a p53-induced Modulator of Autophagy, is Critical for Apoptosis. *Cell* (2006) 126(1):121–34. doi: 10.1016/j.cell.2006.05.034
157. Crighton D, Wilkinson S, Ryan KM. DRAM Links Autophagy to p53 and Programmed Cell Death. *Autophagy* (2007) 3(1):72–4. doi: 10.4161/auto.3438
158. Takahashi M, Kakudo Y, Takahashi S, Sakamoto Y, Kato S, Ishioka C. Overexpression of DRAM Enhances p53-Dependent Apoptosis. *Cancer Med* (2013) 2(1):1–10. doi: 10.1002/cam4.39
159. Green DR, Kroemer G. Cytoplasmic Functions of the Tumour Suppressor P53. *Nature* (2009) 458(7242):1127–30. doi: 10.1038/nature07986
160. Tasdemir E, Maiuri MC, Galluzzi L, Vitale I, Djavaheri-Mergny M, D'Amelio M, et al. Regulation of Autophagy by Cytoplasmic P53. *Nat Cell Biol* (2008) 10(6):676–87. doi: 10.1038/ncb1730
161. Lee SW, Kim HK, Lee NH, Yi HY, Kim HS, Hong SH, et al. The Synergistic Effect of Combination Temozolomide and Chloroquine Treatment is Dependent on Autophagy Formation and p53 Status in Glioma Cells. *Cancer Lett* (2015) 360(2):195–204. doi: 10.1016/j.canlet.2015.02.012
162. Aoki K, Nakamura H, Suzuki H, Matsuo K, Kataoka K, Shimamura T, et al. Prognostic Relevance of Genetic Alterations in Diffuse Lower-Grade Gliomas. *Neuro Oncol* (2018) 20(1):66–77. doi: 10.1093/neuonc/nox132
163. Bäcklund LM, Nilsson BR, Liu L, Ichimura K, Collins VP. Mutations in Rb1 Pathway-Related Genes are Associated With Poor Prognosis in Anaplastic Astrocytomas. *Br J Cancer* (2005) 93(1):124–30. doi: 10.1038/sj.bjc.6602661
164. Mao H, Lebrun DG, Yang J, Zhu VF, Li M. Deregulated Signaling Pathways in Glioblastoma Multiforme: Molecular Mechanisms and Therapeutic Targets. *Cancer Invest* (2012) 30(1):48–56. doi: 10.3109/07357907.2011.630050
165. Delou JM, Biasoli D, Borges HL. The Complex Link Between Apoptosis and Autophagy: A Promising New Role for RB. *Acad Bras Cienc* (2016) 88 (4):2257–75. doi: 10.1590/0001-3765201620160127
166. Polager S, Ofir M, Ginsberg D. E2F1 Regulates Autophagy and the Transcription of Autophagy Genes. *Oncogene* (2008) 27(35):4860–4. doi: 10.1038/onc.2008.117
167. Jiang H, Martin V, Gomez-Manzano C, Johnson DG, Alonso M, White E, et al. The RB-E2F1 Pathway Regulates Autophagy. *Cancer Res* (2010) 70 (20):7882–93. doi: 10.1158/0008-5472.Can-10-1604

168. Jiang H, Martin V, Alonso M, Gomez-Manzano C, Fueyo J. Rb-E2F1: Molecular Rheostat for Autophagy and Apoptosis. *Autophagy* (2010) 6 (8):1216–7. doi: 10.4161/auto.6.8.13695
169. Tracy K, Dibling BC, Spike BT, Knabb JR, Schumacker P, Macleod KF. BNIP3 is an RB/E2F Target Gene Required for Hypoxia-Induced Autophagy. *Mol Cell Biol* (2007) 27(17):6229–42. doi: 10.1128/mcb.02246-06
170. Biasoli D, Kahn SA, Cornélio TA, Furtado M, Campanati L, Chneiweiss H, et al. Retinoblastoma Protein Regulates the Crosstalk Between Autophagy and Apoptosis, and Favors Glioblastoma Resistance to Etoposide. *Cell Death Dis* (2013) 4:e767. doi: 10.1038/cddis.2013.283
171. Liu X, Sun K, Wang H, Dai Y. Knockdown of Retinoblastoma Protein may Sensitize Glioma Cells to Cisplatin Through Inhibition of Autophagy. *Neurosci Lett* (2016) 620:137–42. doi: 10.1016/j.neulet.2016.04.001
172. An Z, Aksoy O, Zheng T, Fan QW, Weiss WA. Epidermal Growth Factor Receptor and EGFRvIII in Glioblastoma: Signaling Pathways and Targeted Therapies. *Oncogene* (2018) 37(12):1561–75. doi: 10.1038/s41388-017-0045-7
173. Bale TA, Jordan JT, Rapalino O, Ramamurthy N, Jessop N, DeWitt JC, et al. Financially Effective Test Algorithm to Identify an Aggressive, EGFR-amplified Variant of IDH-wildtype, Lower-Grade Diffuse Glioma. *Neuro Oncol* (2019) 21(5):596–605. doi: 10.1093/neuonc/noy201
174. Fraser J, Cabodevilla AG, Simpson J, Gammoh N. Interplay of Autophagy, Receptor Tyrosine Kinase Signalling and Endocytic Trafficking. *Essays Biochem* (2017) 61(6):597–607. doi: 10.1042/ebc20170091
175. Sigismund S, Avanzato D, Lanzetti L. Emerging Functions of the EGFR in Cancer. *Mol Oncol* (2018) 12(1):3–20. doi: 10.1002/1878-0261.12155
176. Wu M, Zhang P. EGFR-Mediated Autophagy in Tumourigenesis and Therapeutic Resistance. *Cancer Lett* (2020) 469:207–16. doi: 10.1016/j.canlet.2019.10.030
177. Tini P, Belmonte G, Toscano M, Miracco C, Palumbo S, Pastina P, et al. Combined Epidermal Growth Factor Receptor and Beclin1 Autophagic Protein Expression Analysis Identifies Different Clinical Presentations, Responses to Chemo- and Radiotherapy, and Prognosis in Glioblastoma. *BioMed Res Int* (2015) 2015:208076. doi: 10.1155/2015/208076
178. Eimer S, Belaud-Rotureau MA, Airiau K, Jeanneteau M, Laharanne E, Véron N, et al. Autophagy Inhibition Cooperates With Erlotinib to Induce Glioblastoma Cell Death. *Cancer Biol Ther* (2011) 11(12):1017–27. doi: 10.4161/cbt.11.12.15693
179. Ghildiyal R, Dixit D, Sen E. EGFR Inhibitor BIBU Induces Apoptosis and Defective Autophagy in Glioma Cells. *Mol Carcinog* (2013) 52(12):970–82. doi: 10.1002/mc.21938
180. Jutten B, Keulers TG, Peeters HJM, Schaaf MBE, Savelkouls KGM, Compter I, et al. EgfrvIII Expression Triggers a Metabolic Dependency and Therapeutic Vulnerability Sensitive to Autophagy Inhibition. *Autophagy* (2018) 14(2):283–95. doi: 10.1080/15548627.2017.1409926
181. Sugawa N, Ekstrand AJ, James CD, Collins VP. Identical Splicing of Aberrant Epidermal Growth Factor Receptor Transcripts From Amplified Rearranged Genes in Human Glioblastomas. *Proc Natl Acad Sci USA* (1990) 87 (21):8602–6. doi: 10.1073/pnas.87.21.8602
182. Frederick L, Wang XY, Eley G, James CD. Diversity and Frequency of Epidermal Growth Factor Receptor Mutations in Human Glioblastomas. *Cancer Res* (2000) 60(5):1383–7.
183. Nagane M, Levitzki A, Gazit A, Cavenee WK, Huang HJ. Drug Resistance of Human Glioblastoma Cells Conferred by a Tumor-Specific Mutant Epidermal Growth Factor Receptor Through Modulation of Bcl-XL and caspase-3-like Proteases. *Proc Natl Acad Sci USA* (1998) 95(10):5724–9. doi: 10.1073/pnas.95.10.5724
184. Chakravarti A, Chakladar A, Delaney MA, Latham DE, Loeffler JS. The Epidermal Growth Factor Receptor Pathway Mediates Resistance to Sequential Administration of Radiation and Chemotherapy in Primary Human Glioblastoma Cells in a RAS-dependent Manner. *Cancer Res* (2002) 62(15):4307–15.
185. Gini B, Zanca C, Guo D, Matsutani T, Masui K, Ikegami S, et al. The mTOR Kinase Inhibitors, CC214-1 and CC214-2, Preferentially Block the Growth of EGFRvIII-activated Glioblastomas. *Clin Cancer Res* (2013) 19(20):5722–32. doi: 10.1158/1078-0432.Ccr-13-0527
186. Lee JK, Joo KM, Lee J, Yoon Y, Nam DH. Targeting the Epithelial to Mesenchymal Transition in Glioblastoma: The Emerging Role of MET Signaling. *Onco Targets Ther* (2014) 7:1933–44. doi: 10.2147/ott.S36582
187. Cheng F, Guo D. MET in Glioma: Signaling Pathways and Targeted Therapies. *J Exp Clin Cancer Res* (2019) 38(1):270. doi: 10.1186/s13046-019-1269-x
188. Cruickshanks N, Zhang Y, Yuan F, Pahuski M, Gibert M, Abounader R. Role and Therapeutic Targeting of the HGF/MET Pathway in Glioblastoma. *Cancers (Basel)* (2017) 9(7):87. doi: 10.3390/cancers9070087
189. Lee JS, Oh E, Yoo JY, Choi KS, Yoon MJ, Yun CO. Adenovirus Expressing Dual c-Met-specific shRNA Exhibits Potent Antitumor Effect Through Autophagic Cell Death Accompanied by Senescence-Like Phenotypes in Glioblastoma Cells. *Oncotarget* (2015) 6(6):4051–65. doi: 10.18632/oncotarget.3018
190. Cohen AL, Holmen SL, Colman H. IDH1 and IDH2 Mutations in Gliomas. *Curr Neurol Neurosci Rep* (2013) 13(5):345. doi: 10.1007/s11910-013-0345-4
191. Barthel FP, Johnson KC, Varn FS, Moskalik AD, Tanner G, Kocakavuk E, et al. Longitudinal Molecular Trajectories of Diffuse Glioma in Adults. *Nature* (2019) 576(7785):112–20. doi: 10.1038/s41586-019-1775-1
192. Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, et al. IDH1 and IDH2 Mutations in Gliomas. *N Engl J Med* (2009) 360(8):765–73. doi: 10.1056/NEJMoa0808710
193. Han S, Liu Y, Cai SJ, Qian M, Ding J, Larion M, et al. IDH Mutation in Glioma: Molecular Mechanisms and Potential Therapeutic Targets. *Br J Cancer* (2020) 122(11):1580–9. doi: 10.1038/s41416-020-0814-x
194. Braun Y, Filipski K, Bernatz S, Baumgarten P, Roller B, Zinke J, et al. Linking Epigenetic Signature and Metabolic Phenotype in IDH Mutant and IDH Wildtype Diffuse Glioma. *Neuropathol Appl Neurobiol* (2020) 47(3):379–93 doi: 10.1111/nan.12669
195. Kickingereder P, Sahm F, Radbruch A, Wick W, Heiland S, Deimling A, et al. IDH Mutation Status is Associated With a Distinct Hypoxia/Angiogenesis Transcriptome Signature Which is non-Invasively Predictable With rCBV Imaging in Human Glioma. *Sci Rep* (2015) 5:16238. doi: 10.1038/srep16238
196. Fan Y, Peng X, Li B, Zhao G. Development of Autophagy Signature-Based Prognostic Nomogram for Refined Glioma Survival Prognostication. *BioMed Res Int* (2020) 2020:1872962. doi: 10.1155/2020/1872962
197. Xu Y, Li R, Li X, Dong N, Wu D, Hou L, et al. An Autophagy-Related Gene Signature Associated With Clinical Prognosis and Immune Microenvironment in Gliomas. *Front Oncol* (2020) 10:571189. doi: 10.3389/fonc.2020.571189
198. Binabaj MM, Bahrami A, ShahidSales S, Joodi M, Joudi Mashhad M, Hassanian SM, et al. The Prognostic Value of MGMT Promoter Methylation in Glioblastoma: A Meta-Analysis of Clinical Trials. *J Cell Physiol* (2018) 233(1):378–86. doi: 10.1002/jcp.25896
199. Alnahhas I, Alsawas M, Rayi A, Palmer JD, Raval R, Ong S, et al. Characterizing Benefit From Temozolomide in MGMT Promoter Unmethylated and Methylated Glioblastoma: A Systematic Review and Meta-Analysis. *Neurooncol Adv* (2020) 2(1):vdaa082. doi: 10.1093/noajnl/vdaa082
200. Ryskalin L, Gaglione A, Limanaqi F, Biagioni F, Familiari P, Frati A, et al. The Autophagy Status of Cancer Stem Cells in Gliobastoma Multiforme: From Cancer Promotion to Therapeutic Strategies. *Int J Mol Sci* (2019) 20 (15):3824. doi: 10.3390/ijms20153824
201. Bazzoni R, Bentivegna A. Role of Notch Signaling Pathway in Glioblastoma Pathogenesis. *Cancers (Basel)* (2019) 11(3):292. doi: 10.3390/cancers11030292
202. Takebe N, Nguyen D, Yang SX. Targeting Notch Signaling Pathway in Cancer: Clinical Development Advances and Challenges. *Pharmacol Ther* (2014) 141(2):140–9. doi: 10.1016/j.pharmthera.2013.09.005
203. Yao J, Zheng K, Li C, Liu H, Shan X. Interference of Notch1 Inhibits the Growth of Glioma Cancer Cells by Inducing Cell Autophagy and Down-Regulation of Notch1-Hes-1 Signaling Pathway. *Med Oncol* (2015) 32 (6):610. doi: 10.1007/s12032-015-0610-2
204. Tao Z, Li T, Ma H, Yang Y, Zhang C, Hai L, et al. Autophagy Suppresses Self-Renewal Ability and Tumorigenicity of Glioma-Initiating Cells and Promotes Notch1 Degradation. *Cell Death Dis* (2018) 9(11):1063. doi: 10.1038/s41419-018-0957-3
205. Wu X, Fleming A, Ricketts T, Pavel M, Virgin H, Menzies FM, et al. Autophagy Regulates Notch Degradation and Modulates Stem Cell Development and Neurogenesis. *Nat Commun* (2016) 7:10533. doi: 10.1038/ncomms10533

206. Zhang T, Guo L, Wang Y, Yang Y. Macroautophagy Regulates Nuclear NOTCH1 Activity Through Multiple P62 Binding Sites. *IUBMB Life* (2018) 70(10):985–94. doi: 10.1002/iub.1891
207. Natsumeda M, Maitani K, Liu Y, Miyahara H, Kaur H, Chu Q, et al. Targeting Notch Signaling and Autophagy Increases Cytotoxicity in Glioblastoma Neurospheres. *Brain Pathol* (2016) 26(6):713–23. doi: 10.1111/bpa.12343
208. Lee Y, Lee JK, Ahn SH, Lee J, Nam DH. WNT Signaling in Glioblastoma and Therapeutic Opportunities. *Lab Invest* (2016) 96(2):137–50. doi: 10.1038/labinvest.2015.140
209. Tompa M, Kalovits F, Nagy A, Kalman B. Contribution of the Wnt Pathway to Defining Biology of Glioblastoma. *Neuromolecular Med* (2018) 20(4):437–51. doi: 10.1007/s12017-018-8514-x
210. Zuccarini M, Giuliani P, Ziberi S, Carluccio M, Iorio PD, Caciagli F, et al. The Role of Wnt Signal in Glioblastoma Development and Progression: A Possible New Pharmacological Target for the Therapy of This Tumor. *Genes (Basel)* (2018) 9(2):105. doi: 10.3390/genes9020105
211. Wang T, Chen Z, Zhang W. Regulation of Autophagy Inhibition and Inflammatory Response in Glioma by Wnt Signaling Pathway. *Oncol Lett* (2017) 14(6):7197–200. doi: 10.3892/ol.2017.7103
212. Nàger M, Sallán MC, Visa A, Pushparaj C, Santacana M, Macià A, et al. Inhibition of WNT-CTNNB1 Signaling Upregulates SQSTM1 and Sensitizes Glioblastoma Cells to Autophagy Blockers. *Autophagy* (2018) 14(4):619–36. doi: 10.1080/15548627.2017.1423439
213. Yun EJ, Kim S, Hsieh JT, Baek ST. Wnt/β-Catenin Signaling Pathway Induces Autophagy-Mediated Temozolomide-Resistance in Human Glioblastoma. *Cell Death Dis* (2020) 11(9):771. doi: 10.1038/s41419-020-02988-8
214. Colella B, Faienza F, Carinci M, D'Alessandro G, Catalano M, Santoro A, et al. Autophagy Induction Impairs Wnt/β-Catenin Signalling Through β-Catenin Relocalisation in Glioblastoma Cells. *Cell Signal* (2019) 53:357–64. doi: 10.1016/j.cellsig.2018.10.017
215. Chang L, Zhao D, Liu HB, Wang QS, Zhang P, Li CL, et al. Activation of Sonic Hedgehog Signaling Enhances Cell Migration and Invasion by Induction of Matrix Metalloproteinase-2 and -9 Via the Phosphoinositide-3 Kinase/AKT Signaling Pathway in Glioblastoma. *Mol Med Rep* (2015) 12 (5):6702–10. doi: 10.3892/mmr.2015.4229
216. Clement V, Sanchez P, de Tribolet N, Radovanovic I, Ruiz i Altaba A. Hedgehog-GLI1 Signaling Regulates Human Glioma Growth, Cancer Stem Cell Self-Renewal, and Tumorigenicity. *Curr Biol* (2007) 17(2):165–72. doi: 10.1016/j.cub.2006.11.033
217. Carpenter RL, Lo HW. Hedgehog Pathway and GLI1 Isoforms in Human Cancer. *Discovery Med* (2012) 13(69):105–13.
218. Tang X, Deng L, Chen Q, Wang Y, Xu R, Shi C, et al. Inhibition of Hedgehog Signaling Pathway Impedes Cancer Cell Proliferation by Promotion of Autophagy. *Eur J Cell Biol* (2015) 94(5):223–33. doi: 10.1016/j.ejcb.2015.03.003
219. Carballo GB, Ribeiro JH, Lopes GPF, Ferrer VP, Dezonne RS, Pereira CM, et al. GANT-61 Induces Autophagy and Apoptosis in Glioblastoma Cells Despite Their Heterogeneity. *Cell Mol Neurobiol* (2020). doi: 10.1007/s10571-020-00891-6
220. Honorato JR, Hauser-Davis RA, Saggioro EM, Correia FV, Sales-Junior SF, Soares LOS, et al. Role of Sonic Hedgehog Signaling in Cell Cycle, Oxidative Stress, and Autophagy of Temozolomide Resistant Glioblastoma. *J Cell Physiol* (2020) 235(4):3798–814. doi: 10.1002/jcp.29274
221. Chen X, Morales-Alcala CC, Riobo-Del Galdo NA. Autophagic Flux is Regulated by Interaction Between the C-terminal Domain of PATCHED1 and ATG101. *Mol Cancer Res* (2018) 16(5):909–19. doi: 10.1158/1541-7786.Mcr-17-0597
222. Marjanovic Vicentic J, Drakulic D, Garcia I, Vukovic V, Aldaz P, Puskas N, et al. SOX3 can Promote the Malignant Behavior of Glioblastoma Cells. *Cell Oncol (Dordr)* (2019) 42(1):41–54. doi: 10.1007/s13402-018-0405-5
223. Stupp R, Taillibert S, Kanner AA, Kesari S, Steinberg DM, Toms SA, et al. Maintenance Therapy With Tumor-Treating Fields Plus Temozolomide vs Temozolomide Alone for Glioblastoma: A Randomized Clinical Trial. *JAMA* (2015) 314(23):2535–43. doi: 10.1001/jama.2015.16669
224. Escamilla-Ramírez A, Castillo-Rodríguez RA, Zavala-Vega S, Jimenez-Farfan D, Anaya-Rubio I, Briseño E, et al. Autophagy as a Potential Therapy for Malignant Glioma. *Pharmaceuticals (Basel)* (2020) 13(7):156. doi: 10.3390/ph13070156
225. Zhang X, Deibert CP, Kim WJ, Jaman E, Rao AV, Lotze MT, et al. Autophagy Inhibition is the Next Step in the Treatment of Glioblastoma Patients Following the Stupp Era. *Cancer Gene Ther* (2020). doi: 10.1038/s41417-020-0205-8
226. Sotelo J, Briceño E, López-González MA. Adding Chloroquine to Conventional Treatment for Glioblastoma Multiforme: A Randomized, Double-Blind, Placebo-Controlled Trial. *Ann Intern Med* (2006) 144 (5):337–43. doi: 10.7326/0003-4819-144-5-200603070-00008
227. Briceño E, Calderon A, Sotelo J. Institutional Experience With Chloroquine as an Adjuvant to the Therapy for Glioblastoma Multiforme. *Surg Neurol* (2007) 67(4):388–91. doi: 10.1016/j.surneu.2006.08.080
228. Rosenfeld MR, Ye X, Supko JG, Desideri S, Grossman SA, Brem S, et al. A Phase I/II Trial of Hydroxychloroquine in Conjunction With Radiation Therapy and Concurrent and Adjuvant Temozolomide in Patients With Newly Diagnosed Glioblastoma Multiforme. *Autophagy* (2014) 10(8):1359–68. doi: 10.4161/auto.28984
229. Compter I, Eekers DBP, Hoeben A, Rouschop KMA, Reymen B, Ackermans L, et al. Chloroquine Combined With Concurrent Radiotherapy and Temozolomide for Newly Diagnosed Glioblastoma: A Phase IB Trial. *Autophagy* (2020) 1–9. doi: 10.1080/15548627.2020.1816343

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Jandrey, Bezerra, Inoue, Furnari, Camargo and Costa. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 03 August 2021
doi: 10.3389/fcell.2021.650846

Blockage of Extracellular Signal-Regulated Kinase Exerts an Antitumor Effect via Regulating Energy Metabolism and Enhances the Efficacy of Autophagy Inhibitors by Regulating Transcription Factor EB Nuclear Translocation in Osteosarcoma

*Man Zhang[1,2,3], Yang Bai[4], Chang Xu[4], Yiying Qi[1], Jiahong Meng[1], Wenkan Zhang[1], Hang Su[1] and Weiqi Yan[1,2,3]**

[1] Department of Orthopedic Surgery, The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China, [2] Orthopedics Research Institute of Zhejiang University, Hangzhou, China, [3] Key Laboratory of Motor System Disease Research and Precision Therapy of Zhejiang Province, Hangzhou, China, [4] The Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China

OPEN ACCESS

Edited by:
Daniel Hector Grasso, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

Reviewed by:
Rajesh Singh, Maharaja Sayajirao University of Baroda, India
Mengxiong Sun, Shanghai General Hospital, China

***Correspondence:**
Weiqi Yan
wyan@zju.edu.cn

Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: 08 January 2021
Accepted: 15 March 2021
Published: 03 August 2021

Citation:
Zhang M, Bai Y, Xu C, Qi Y, Meng J, Zhang W, Su H and Yan W (2021) Blockage of Extracellular Signal-Regulated Kinase Exerts an Antitumor Effect via Regulating Energy Metabolism and Enhances the Efficacy of Autophagy Inhibitors by Regulating Transcription Factor EB Nuclear Translocation in Osteosarcoma. Front. Cell Dev. Biol. 9:650846. doi: 10.3389/fcell.2021.650846

Accumulating evidence suggests that extracellular signal-regulated kinase (ERK) is a valuable target molecule for cancer. However, antitumor drugs targeting ERK are still in their clinical phase and no FDA-approved medications exist. In this study, we identified an ERK inhibitor (ERKi; Vx-11e) with potential antitumor activities, which was reflected by the inhibition in the survival and proliferation of Osteosarcoma (OS) cells. Mechanistically, the ERKi regulated autophagic flux by promoting the translocation of transcription factor EB (TFEB) in OS cells, thereby increasing the dependence of OS cells on autophagy and sensitivity to treatment with autophagy inhibitors in OS. Besides, we also found that the ERKi could regulate mitochondrial apoptosis through the ROS/mitochondria pathway and aerobic glycolysis in OS, which also increases the dependence of OS cells on autophagy to clear metabolites to a certain extent. These results may provide a reference for the clinically improved efficacy of ERKis in combination with autophagy inhibitors in the treatment of OS and indicate its potential as a therapeutic agent.

Keywords: ERK, transcription factor EB, autophagy, osteosarcoma, energy metabolism

INTRODUCTION

Osteosarcoma (OS) is a highly malignant bone tumor characterized by early metastasis. The incidence of OS is rising rapidly, and it is known to have the highest mortality rate among all cancer types, especially in children and adolescents (Zhang, 2019). With the development of several therapies such as surgery, chemotherapy, radiation, targeted therapy, and immunotherapy, the survival rate of OS patients has increased; however, this rate still is unsatisfactory

(Ritter and Bielack, 2010). There are several problems that need to be overcome among the current treatment strategies, such as effective inhibition of the metastasis, and the reduction in severe side effects and drug resistance caused by single-drug chemotherapy. Therefore, it is necessary to explore new treatment methods and strategies to treat OS. Particularly, therapies focusing on combination therapy are urgently needed.

As an important cellular recycling mechanism, autophagy is responsible for the degradation of unnecessary or dysfunctional proteins and organelles within cells (Glick et al., 2010). Autophagy promotes the growth and survival of various cancer cells as it increases their ability to support cellular metabolism. Studies have shown that activation of autophagy is beneficial for cellular growth and survival in breast cancer (Wei et al., 2011), glioblastoma (Jawhari et al., 2016), and hematological malignancies (Karvela et al., 2016) by the maintenance of glycolytic capacity. Autophagy restricts oxidative stress, prevents intra-tumoral necrosis and local inflammation in response to stress, and regulates glycolysis to promote tumor growth (Mowers et al., 2018). However, in OS, autophagy acts as both a pro-tumoral and antitumoral process. A previous study suggests that autophagy facilitates glycolytic metabolism and increases lung colonization by OS cells *via* upregulating the expression of intercellular adhesion molecule-1 (ICAM-1) and enhancing tumor metastasis (Itoh et al., 2018). Another study has shown that autophagy induces cell apoptosis in OS (Meschini et al., 2008). Based on these studies, the role of autophagy in the growth of OS cells poses several challenges that need to be further explored. Transcription factor EB (TFEB) is a subfamily of the MiT/TFE basic helix–loop–helix leucine-zipper (bHLH-Zip) family of transcription activators (Sardiello, 2016). TFEB regulates various cellular processes, including autophagy (Martini-Stoica et al., 2016), lysosomal biogenesis (Chao et al., 2018), and energy metabolism (Pastore et al., 2017). TFEB activity is tightly controlled through TFEB nuclear translocation. Multiple environmental stimuli, including proinflammatory agents, mitochondrial and oxidative stress, can promote TFEB nuclear translocation to increase its transcription activity. However, its mechanistic interaction with autophagy in OS remains elusive.

Mitochondria are the primary sites of energy metabolism in eukaryotic cells that provide energy for cell growth, proliferation, and other biological activities (Horbay and Bilyy, 2016). However, given that the "Warburg effect" is one of the basic characteristics of tumor cells, the activity in mitochondria is also necessary for many tumor cells (Hsu et al., 2016). Additionally, mitochondria control calcium homeostasis and redox reactions and participate in transcriptional regulation (Hsu et al., 2016; Porporato et al., 2018). Moreover, a dysregulated mitochondrial respiratory chain or exposure to certain chemicals affects the mitochondrial DNA in cancer cells, thereby inducing mitochondrial dysfunction and promoting cancer progression to a chemoresistant or invasive phenotype (Giang et al., 2013; Hsu et al., 2016). Therefore, mitochondria are a potential target for the development of new anticancer drugs. Mitochondria exist in dynamic equilibrium by continuous fusion and fission events. During this process, mitochondrial morphology changes, resulting in the elimination of damaged mitochondria and the production of new ones (Horbay and Bilyy, 2016). Studies have shown that the induction of mitochondrial dysfunction in OS cells is associated with the modulation of mitochondrial fission/fusion proteins that exert anticancer effects (Huang et al., 2014). However, another study shows that the suppression of mitochondrial function contributes to the Warburg effect in OS cells (Giang et al., 2013; Gorska-Ponikowska et al., 2018). Hence, the role of mitochondria in OS remains slightly controversial and needs to be further explored. Mitogen-activated protein kinases (MAPKs) regulate the growth, proliferation, and apoptosis of cells (Cuadrado and Nebreda, 2010; Santos and Crespo, 2018), and the excessive activation of MAPKs is found in several tumors (Wagner and Nebreda, 2009; Yong et al., 2009). Extracellular signal-regulated kinase (ERK)1/2 is one of the main members of the MAPK family (Kim and Choi, 2010). The expression level of ERK is increased in various human tumors, and interferences with the ERK pathway may be a potential therapeutic strategy for treating these cancers (Samatar and Poulikakos, 2014; Santos and Crespo, 2018). Activation of the ERK pathway has been confirmed to promote autophagy in cancer cells, and some literature suggests that the inhibition of ERK inhibits aerobic glycolysis and induces stress, leading to the death of cancer cells (Marchetti et al., 2018; Aloia et al., 2019). However, similar findings have rarely been reported in OS. Therefore, whether ERK has an effect on the metabolism in OS is not fully understood and therefore warrants the elucidation of its underlying mechanism.

MATERIALS AND METHODS

Cell Culture and siRNA Transfection

The human OS cell lines (HOSs), U2-OS (U2), 143B, and MG-63, and the murine spontaneous OS cell line K7M2 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China[1]) and maintained at 37°C and 5% CO_2 in a humid environment. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum and antibiotics (1% penicillin/streptomycin). TFEBsiRNA was delivered into the OS cell line using Lipofectamine 2000. TFEB siRNA was constructed by TSINGKE (Beijing, China). The sequences were as follows: TFEBsiRNA1, F:GAUGUCAUUGACAACAUUATT; R:UAAUGUUGUCAAUGACAUCTT and TFEBsiRNA2, F:CC AAGAAGGAUCUGGACCUTT; R:AGGUCCAGAUCCUUCU UGGTT. Vx-11e (APExBiO) was dissolved in dimethyl sulfoxide (DMSO), and the NC group (DMSO) was treated with the same amount of DMSO. Hydroxychloroquine (HCQ) was purchased from Aladdin. LysoTracker was obtained from Solarbio. Lactic Acid assay kit and Glucose Uptake assay kit were obtained from Nanjing JianCheng Bioengineering Institute.

Cell Proliferation and Cytotoxicity Assays

Cells were seeded into a 96-well plate and treated with different drugs. After 24 h of coculture, the Cell Counting Kit-8 (Dojindo,

[1]http://www.cellbank.org.cn

Japan) dye was added. For colony formation, cells were seeded into a six-well plate at a density of 500–1,000 cells/well. The plate was then shaken well to maintain sufficient distance between individual cells and incubated under different treatment conditions for 14–21 days. The colonies were visualized under a microscope and photographed.

Cell Invasion and Migration Assays

The wound-healing assay was used to detect cell migration. A wound was made using a pipette tip when the cell proliferation reached approximately 80% confluence in the six-well plate. The cells were then visualized and photographed. Transwell chambers (Corning Life Science, United States) were used to determine cell invasion and migration abilities.

Determination of Glutathione Peroxidase (GPx), Glutathione (GSH), and Reactive Oxygen Species (ROS)

Oxidative conversion of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Beyotime Biotech) was used to investigate the levels of intracellular ROS. The detailed procedure was performed as per the manufacturer's instructions. Briefly, the cells were plated in a 6-well plate and subjected to different drug treatments for 6 h and 24 h and then incubated with DCFH-DA for 15 min. Next, the cells from each well were detected using fluorescence microplate assay. Samples were harvested for the measurement of GPx and GSH content spectrophotometrically using the respective assay kits (Beyotime Biotech Inc., Jiangsu, China).

Mitochondrial Transmembrane Potential Assay

To detect the loss of mitochondrial transmembrane potential ($\Delta\Psi m$), the fluorescent probe, JC-1 (Beyotime, Jiangsu, China), was used. Briefly, the cells were seeded in six-well plates, stained with JC-1 staining solution for 15 min at 37°C, and washed with phosphate-buffered saline (PBS). Thereafter, images were obtained using fluorescence microscopy (Olympus).

Transmission Electron Microscopy (TEM) Analysis

Cells were collected and fixed in 2.5% glutaraldehyde for 24 h at 4°C, and the intracellular and morphological changes in the subcellular ultrastructure were investigated by TEM. Next, the cells were again fixed with 1% osmium tetroxide for 2 h at room temperature and then dehydrated in different concentrations and gradients of alcohol. The sample was then finally embedded in Spurr's resin to form pellets. Ultrastructural analysis was performed using TEM (Hitachi H-7650, Japan).

Flow Cytometry

Flow cytometry was performed as per the manufacturer's protocols. Thereafter, the cells were harvested and stained with Annexin V conjugated with APC and 7-aminoactinomycin D (Multi-Sciences, China). Next, FACScan flow cytometry (Becton-Dickinson, United States) was used to analyze the samples and measure the percentage of apoptotic cells.

Glycolysis Measurement

Glucose Uptake Assay Kit and Lactic Acid Assay Kit were used to measure glucose utilization and glycolysis, respectively. Mitochondrial respiration was tested by measuring the oxygen consumption rate (OCR) in OS cells. In brief, cells were added to a 2-mL chamber ($3–4 \times 10^6$ cells/chamber). The oxygen flux (pmol O_2/min) of cells is proportional to oxygen consumption, which was recorded continuously using oxygraphy (Oroboros Oxygraph-2k). Data were analyzed using one-way ANOVA and Student's *t*-test in DatLab software 7.

In vivo Experiments

All animal experiments complied with the guidelines and followed a protocol approved by the Research Ethics Committee of Zhejiang University, China. Nude mice (4-weeks-old, male, Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China) were raised under specific pathogen-free conditions. The mice were inoculated with 2×10^6 K7M2 cells via the marrow cavity of the right tibia. After 7 days, these mice were randomized into three groups. The mice received daily ERKi (50 mg/kg, p.o) and HCQ (60 mg/kg, p.o). Tibial tumors were harvested 2 weeks after treatment, and each tumor was weighed. The tumor sizes were calculated as volume (cm^3) = [$\pi \times$ width^2 (cm^2) $\times$ length (cm)]/6.

Western Blotting and Co-immunoprecipitation (IP)

Cellular proteins (60 μg) were separated using 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories). After blocking (5% fat-free milk) for 1.5 h at room temperature, the membranes were incubated with the following primary antibodies: active-caspase-3 (1:1,000, Cst), Bcl-XL (1:1,000, Cst), C-MYC (1:1,000, Proteintech Group, Inc), Bax (1:500, bioss), Beclin-1 (1:500, Bioss), LC3 (1:1,000, Proteintech Group, Inc), ERK (1:1,000, Cst), p-ERK (1:1,000, Cst), and GAPDH (Cat: RT1210-1, Huabio) overnight at 4°C. The membranes were then washed with TBST and incubated with a secondary antibody for 1 h at room temperature. The signals were visualized with the ChemiDoc^{TM} XRS + Imaging System (BioRad Laboratories, Hercules, CA, United States), and the densities of the immunoreactive bands were analyzed using ImageJ software (NIH, Bethesda, MD, United States). For IP, the commercial antibody was added to whole-cell lysates (WCLs), followed by co-incubation at 4°C for 12 h. Subsequently, the protein A/G agarose beads were added to the cell lysate and co-incubated at 4°C for 2 h. Western blotting was performed to detect the precipitated and co-precipitated proteins.

Immunofluorescence

Cells were placed on a coverslip and fixed with 4% paraformaldehyde at room temperature. After blocking with 5%

bovine serum albumin (BSA) in PBS for 30 min, the samples were incubated with the following primary antibodies: active-caspase-3 (1:100, CST), active-caspase-9 (1:100, CST), LC3B (1:300, Proteintech Group, Inc), and Mff (1:300, Proteintech Group, Inc) at 4°C overnight. After washing three times with PBS, the samples were incubated with the secondary antibody (1:1,000, Huabio), and the sections were then re-stained with anti-fluorescent quench sealant (Yeasen). The fluorescence images were captured using confocal laser microscopy (Nikon, A1PLUS, Tokyo, Japan).

Hematoxylin and Eosin (H&E)

Tissues were collected and fixed in 4% (w/v) paraformaldehyde for 24 h and embedded in paraffin wax. Sections were then prepared and mounted on slides for histopathological examination. H&E staining and Cresyl violet staining were performed according to the manufacturer's instructions. Images were obtained using light microscopy.

Statistical Analysis

Data are presented as means ± SEM. Differences between two groups were determined using Student's *t*-test, whereas one-way ANOVA followed by Dunnett's *post hoc* test was used to determine differences among multiple groups. A $p < 0.05$ was considered significant.

RESULTS

ERK Inhibition Elevates Autophagic Flux Through TFEB

To determine the effect of ERK on the growth of OS cells, we treated OS cells with an ERK-selective inhibitor, Vx-11e (3 μM). As shown in **Figure 1A**, Vx-11e restrained the phosphorylation of ERK in the OS cell line, resulting in an increase in the conversion of LC3 from LC3-I to LC3-II (**Figures 1C,D**). Results from western blotting showed that the expression of Beclin-1 protein was increased after Vx-11e treatment (**Figure 1B**), and the knockdown of TFEB by small interfering RNA reversed the expression of Beclin-1 protein (**Figures 1G,I**); however, bafilomycin A_1 ($BafA_1$, 100 nM), a lysosomal function inhibitor, increases the expression of the protein (**Figure 1B**). The lysosomes expand in Vx-11e-treated cells, as indicated by the lysosome-associated membrane protein 1 (Lamp1) and LysoTracker staining, but not in TFEB-knockdown cells (**Figures 1E,F**). Further, we also found that Vx-11e treatment triggered the nuclear translocation of TFEB (**Figures 1H,J,K**) and abolished the 14-3-3/TFEB complex formation in OS cells (**Figure 1L**).

ERK Inhibition Restrains the Activity of OS Cells

The survival rate of OS cells treated with ERKi gradually decreased in a time-dependent manner (**Figure 2A**). Cellular apoptosis was detected using flow cytometry after annexin V-APC/7-AAD (MultiSciences, China) double staining. The apoptotic abilities of OS cells were significantly higher than those of NC cells (**Figure 2B**). We obtained similar results for the expression of cleaved caspase-3, cleaved poly-ADP-ribose polymerase (PARP), and PARP protein levels using western blotting and immunofluorescence assay (**Figures 2C,E,G**). The invasion and migration of the ERKi groups were significantly abrogated in OS cells compared with those in NC cells (**Figures 2D,F,H**), which was similar to the results observed in the wound-healing assay (**Figure 2I**).

ERK Inhibition Impairs Mitochondrial Activity

The level of ROS in OS cells increased evidently when the cells were exposed to ERKi for an extended duration (**Figures 3A,B**). As shown in **Figures 3C–F**, at 24 h post-treatment, NC cells stained with JC-1 emitted red fluorescence and some green fluorescence; compared to this group, the ERKi-treated group produced more green fluorescence, showing an increased green/red fluorescence ratio. ERKi also suppressed the amount of intracellular GSH and the activity of glutathione peroxidase (GPX) in OS cells (**Figure 3G**), and GSH precursor antioxidant NAC and mitochondrial membrane PT pore inhibitor cyclosporine A (CsA, 5 μM) pre-treatment in OS cells significantly reversed ERKi-induced cell death (**Figure 3I**). Compared with the control group, the treatment group showed spotty fluorescent aggregation of the mitochondria (**Figure 3H**). Microstructural changes in the mitochondria of OS cells observed using TEM showed that in the early stages of ERKi treatment, numerous mitochondria were present that were small in size. With an extension in treatment with ERKi, the number of mitochondria decreased, edema occurred, and mitochondrial ridges became blurred (**Figure 4A**). The expression levels of Bax (**Figures 4B,F**) and active-caspase9 protein (**Figures 4C,D**) were significantly upregulated during ERKi treatment, and the expression of BCL-XL protein showed the opposite trend (**Figures 4B,E**); in contrast, no such difference was observed in the early stage of OS cell treatment with ERKi (**Figures 4B,E**). Our data also showed that ERKi suppressed the aerobic glycolytic capacity (**Figure 4J**) and induced a decrease in c-myc protein level in OS cells (**Figure 4I**). Besides, ERKi does not affect the oxygen consumption of OS cells in the early stages as reflected by OCR measurement (**Figures 4G,H**). In summary, ERKi induced mitochondrial apoptosis and abnormal glycolysis in OS cells. In the early stage, OS cells demonstrate an energy intake disorder. Thus, there were compensatory increases in mitochondrial activity, but no productivity was found.

Effect of HCQ on OS Cells

To explore the effect of HCQ on OS cells, we examined cellular morphology and survival. The survival rate of OS cells treated with HCQ gradually decreased in a concentration-dependent manner (**Figure 5B**). The morphological changes in cells were consistent with the results of survival rate. With an increase in HCQ concentration, the morphology of OS cells shrank and became deformed (**Figure 5A**). The morphology of OS cells was observed using TEM. The number of autophagosomes was

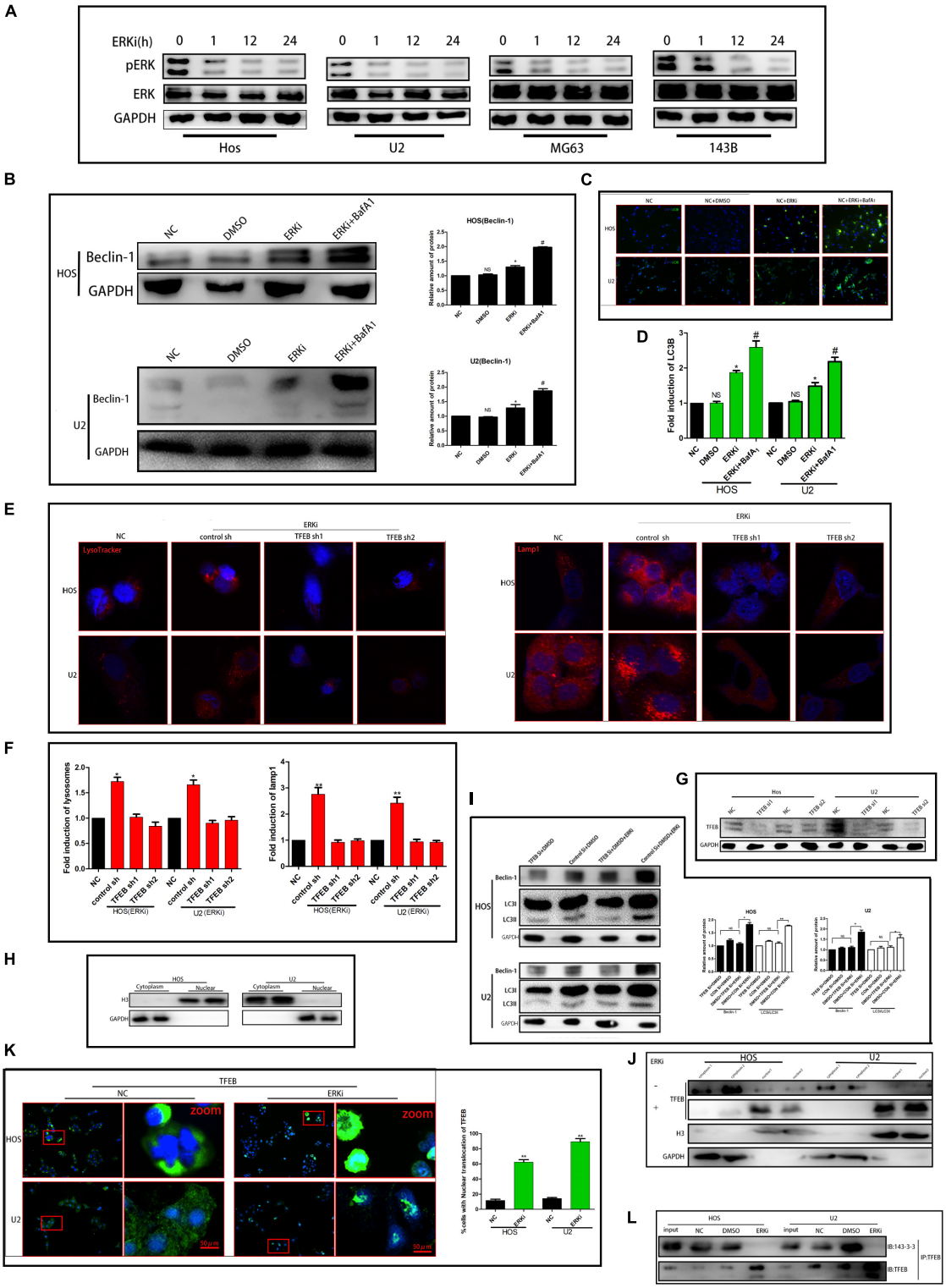


FIGURE 1 | ERKi treatment increases the autophagic flux through TFEB in OS cells. **(A)** Protein expression of p-ERK and ERK after ERKi treatment. **(B)** Protein expression and densitometric quantification of beclin-1 after ERKi and bafilomycin A1 treatment. GAPDH was used as a loading control and for band density normalization. **(C,D)** Immunofluorescence staining of LC3B (green) in OS cells 24 h after ERKi treatment. **(E,F)** Immunofluorescence staining of mitochondria. **(G–I)** Western blotting. **(K)** Immunofluorescence staining of TFEB. **(J)** Immunoblots for TFEB in the cytoplasmic/nuclear fractions of OS cells treated with ERKi. **(L)** ERKi disrupts TFEB interaction with 14–3–3. WCLs of OS cells immunoprecipitated (IP) with anti-TFEB, followed by immunoblotting (IB) with antibodies against 14–3–3 and TFEB ($n = 5$ per group; $^{**}p < 0.01$ versus NC group; $^{*}p < 0.05$ versus NC group).

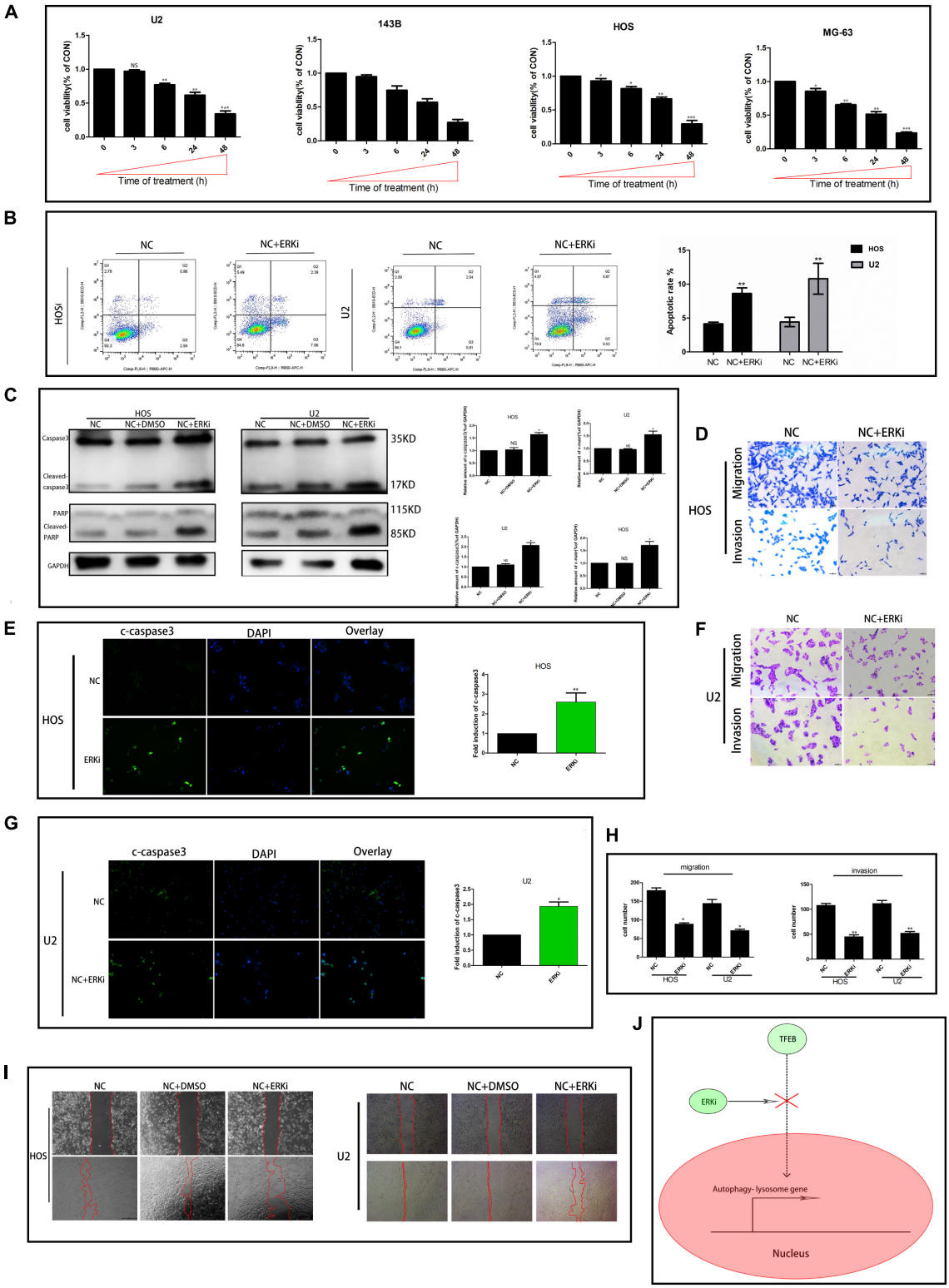


FIGURE 2 | ERK inhibition restrains the activity of OS cells. **(A)** CCK8 proliferation analysis. **(B)** Flow cytometry to determine the total proportion of apoptotic cells in OS. **(C)** Protein expression of caspase3, c-caspase3, c-PARP, and PARP after treatment with ERKi. GAPDH was used as a loading control and for band density normalization. **(E,G)** Immunofluorescence staining of c-caspase3 (green) in OS cells 24 h after ERKi treatment. **(D,F,H)** Migration and invasion. **(I)** Wound-healing assay in different groups. **(J)** Schematic representation of ERKi effect on autophagy in OS cells. Scale bar = 50 μm ($n = 5$ per group; $^{***}p < 0.001$ versus NC group; $^{**}p < 0.01$ versus NC group; $^{*}p < 0.05$ versus NC group).

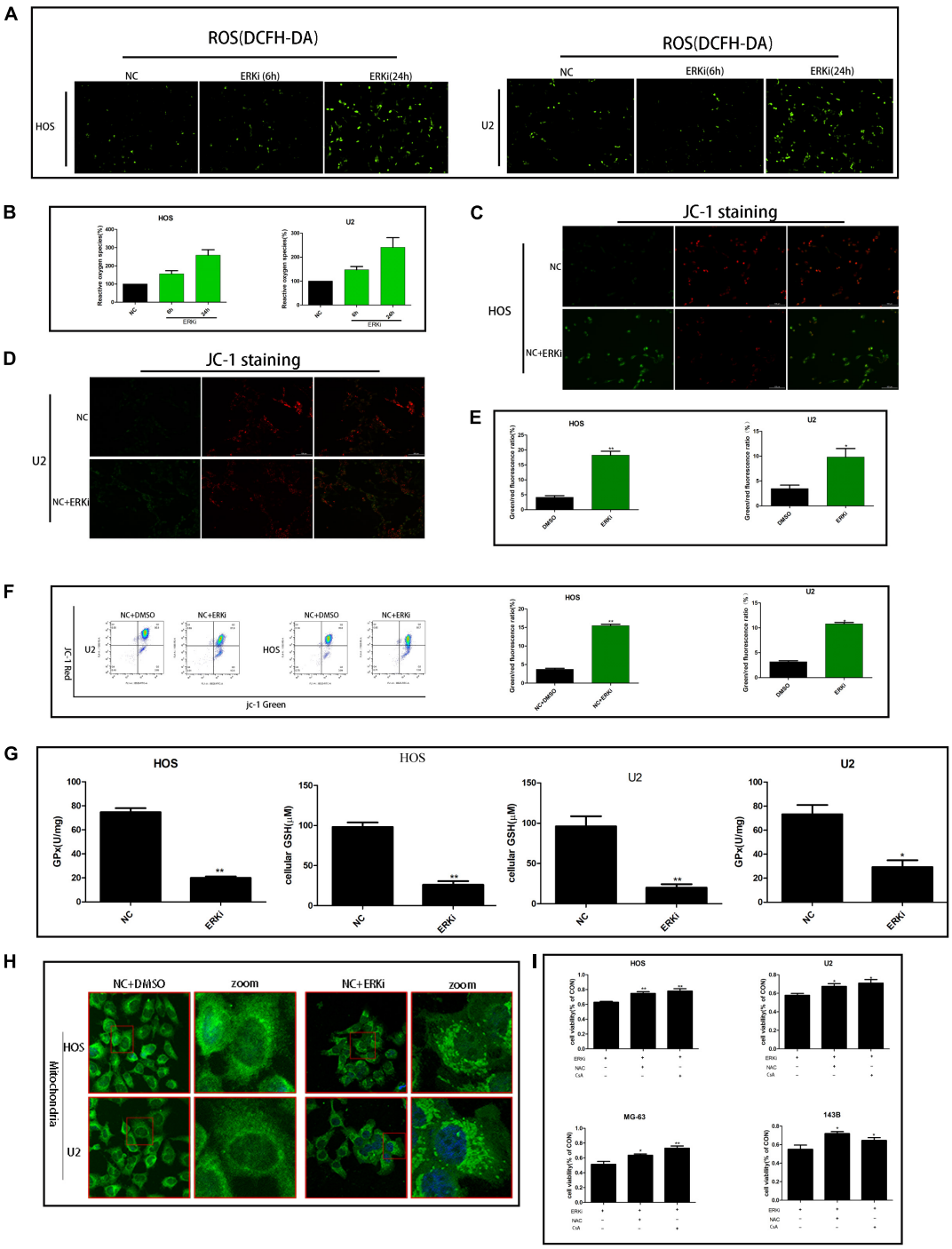


FIGURE 3 | ERK inhibition regulates the mitochondrial activity of OS cells. **(A,B)** ROS fluorescence probe analysis. **(C–E)** JC-1 staining for comparing mitochondrial depolarization of OS cell lines among groups**. (F)** Flow cytometry was used to measure JC-1 of OS cells. **(G)** Effect of ERKi on GPx and GSH levels in OS cells. **(H)** Immunofluorescence co-staining of mitochondria in OS cells 24 h post-ERKi treatment **(I)** Cell-survival rate in different groups ($n = 5$ per group; $^{**}p < 0.01$ versus NC group; $^{*}p < 0.05$ versus NC group).

significantly increased after treatment with HCQ compared with the number in the NC group (**Figure 5E**). Simultaneously, we also found that the number of lysosomes did not decrease. Moreover, we performed immunofluorescence co-staining analysis of autophagy-related proteins LC3B and Lamp-1 in OS cells. Our findings indicated that co-localization of LC3B and Lamp-1 proteins was significantly reduced in the HCQ-treated OS cells compared with the untreated cells (**Figures 5C,D**). Furthermore,

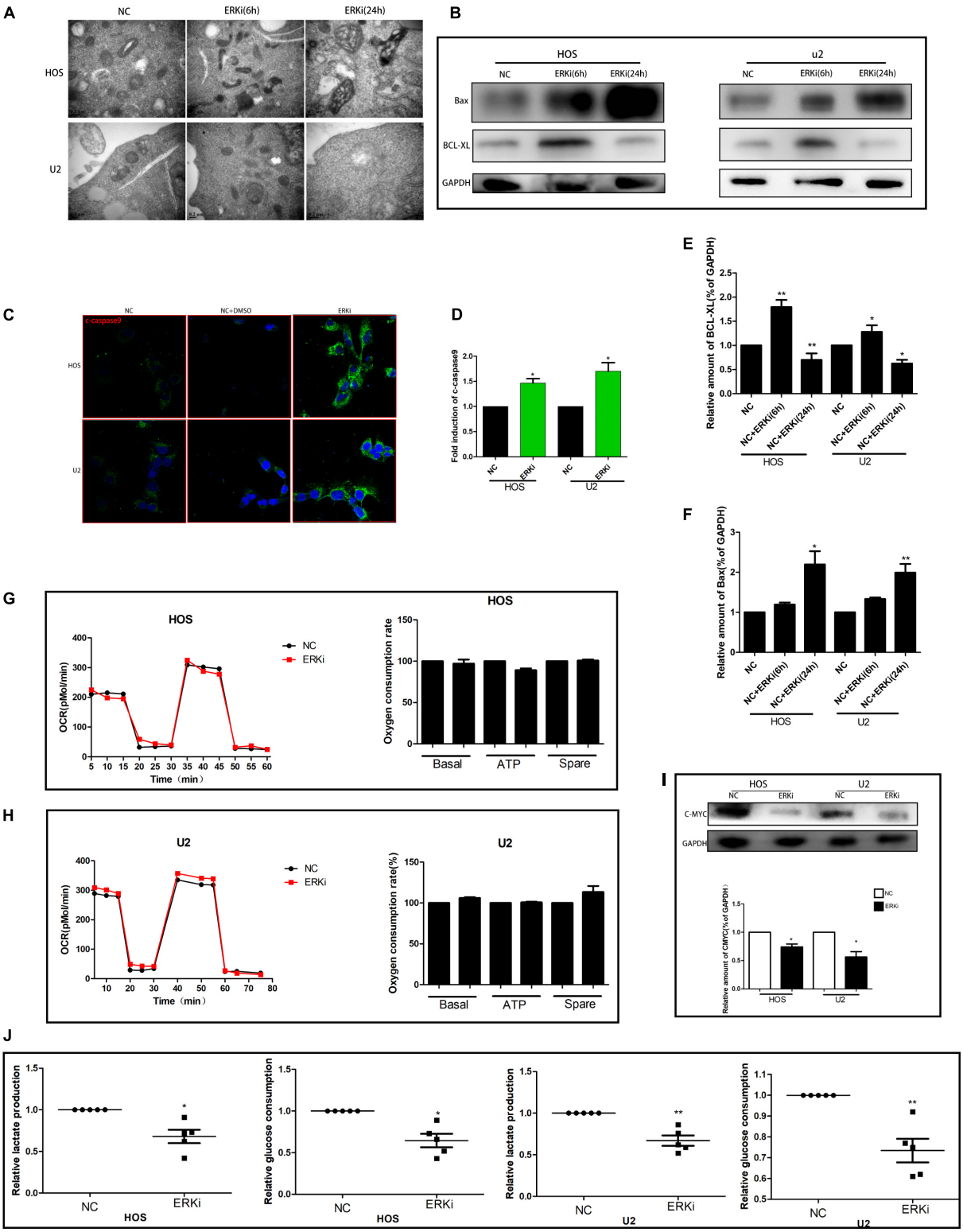


FIGURE 4 | ERK inhibition promotes apoptosis and regulates aerobic glycolysis in OS cells. **(A)** TEM images of ERKi-treated OS cells after 6 h and 24 h of treatment (black arrows). **(B)** Protein expression of Bax and BCL-XL after treatment with ERKi; GAPDH was used as the loading control and for band density normalization. **(C,D)** Immunofluorescence staining of c-caspase9 after 24 h of treatment with ERKi. **(E,F)** Optical density of Bax and BCL-XL proteins. **(G,H)** Diagram of OCR results obtained by OROBOROS Oxygraph-2k. **(I)** Western blotting and densitometric quantification. **(J)** Glucose uptake and production of lactic acid in OS cell lines in different groups ($n = 5$ per group; $^{**}p < 0.01$ versus NC group; $^{*}p < 0.05$ versus NC group).

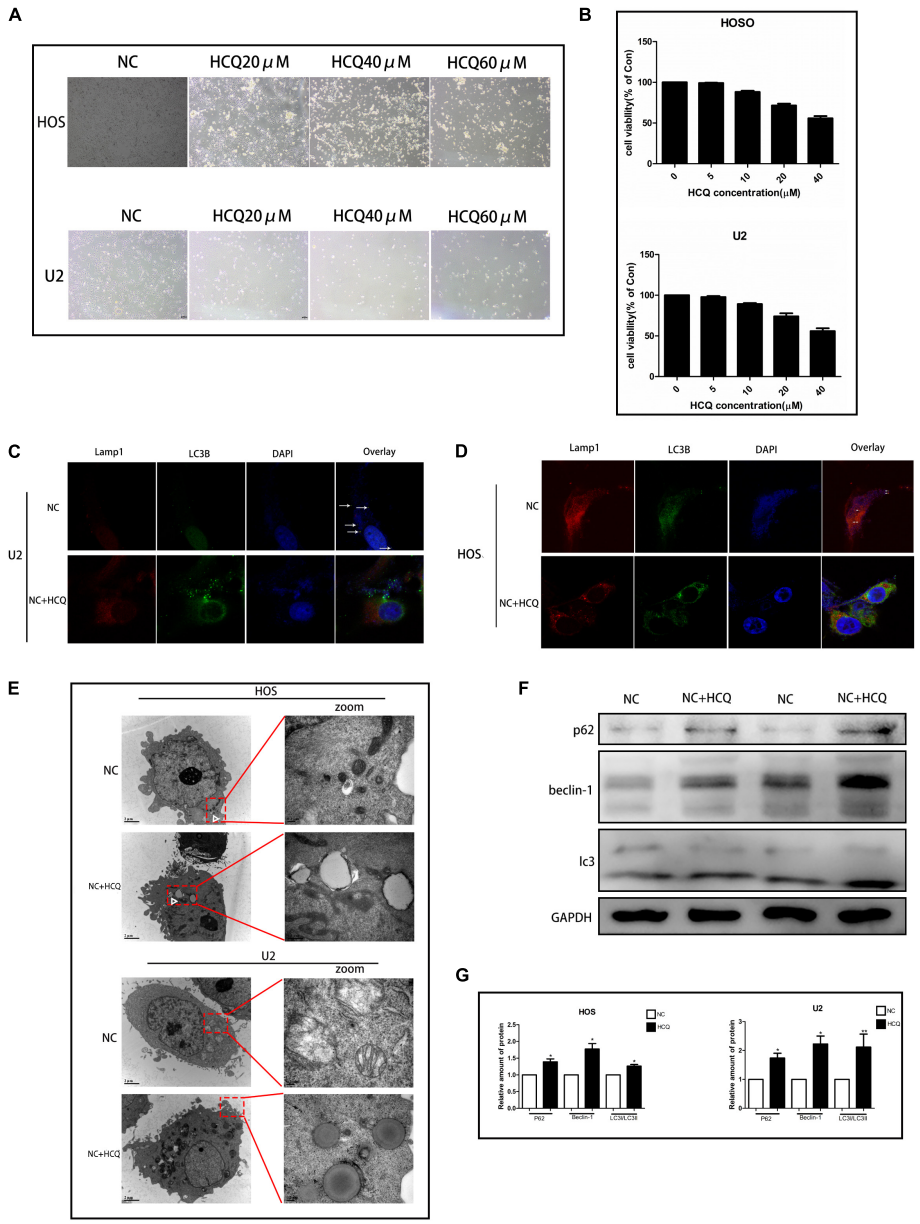


FIGURE 5 | HCQ inhibits the activity of OS cells. **(A)** Cell morphology. **(B)** CCK8 proliferation analysis. **(C,D)** Immunofluorescence co-staining of Lamp-1 (red) and LC3B (green) in OS cells 24 h post-HCQ treatment. **(E)** TEM images of HCQ-treated OS cells after 6 h and 24 h. Autophagosomes are indicated by white arrows. **(F,G)** Western blotting and densitometric quantification ($n = 5$ per group; $^{**}p < 0.01$ versus NC group; $^{*}p < 0.05$ versus NC group).

HCQ treatment increased the expression of LC3B and Beclin-1 proteins (**Figures 5F,G**) in early autophagy.

Combination of ERKi and HCQ Synergistic Inhibition of OS Cell Activity

To verify the synergistic inhibition of OS cell activity by HCQ and ERKi, the growth, proliferation, migration, and apoptosis of OS cells were investigated. In CCK8 analysis, compared with OS cells treated with ERKi alone, the combined use of HCQ had a more pronounced inhibitory effect on OS cell growth (**Figure 6A**). The synergism or antagonism of the drug combination of ERKi and HCQ in OS was analyzed using CompuSyn software. The combination of ERKi and HCQ in OS indicated a favorable dose reduction (**Figures 6B,D**) and played a synergistic role (**Figures 6C,E**). To further characterize the highly tumorigenic capacity of OS cells, we conducted a colony-formation assay. A large and well-spread colony had formed in the NC group. Further, ERKi restrained colony formation, but the combination of ERKi and HCQ had a more potent antitumor effect than ERKi alone (**Figure 6J**). Furthermore, proliferation results indicated that the combination of ERKi and HCQ had more inhibitory

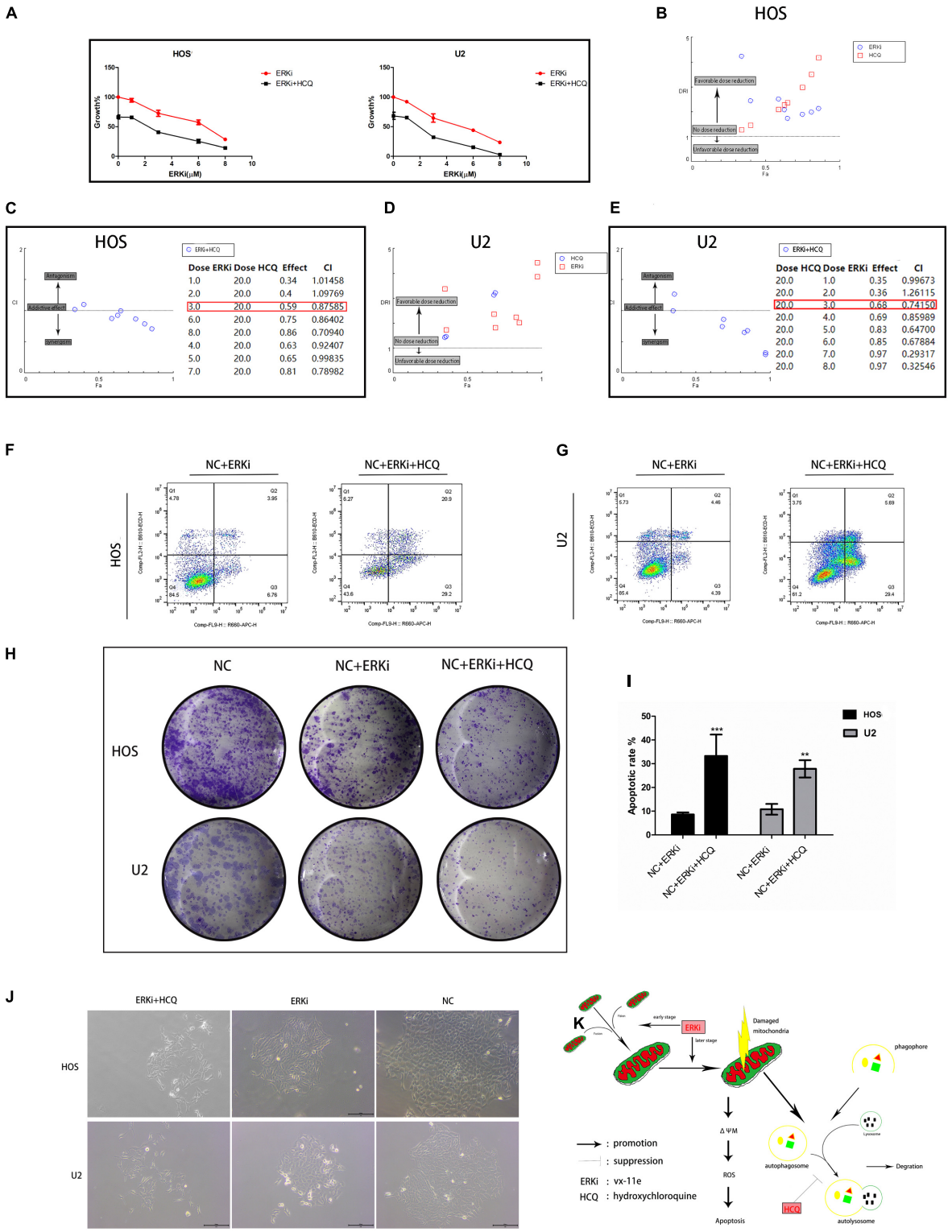


FIGURE 6 | Synergistic inhibitory effect of ERKi and HCQ combined treatment on OS cell activity. **(A)** OS cells were treated with ERKi and HCQ (20 μM) for 24 h. Proliferation was assessed using live-cell counting. (**B,D**) Dose reduction index (DRI) for each drug for a given effect (Fa). DRI of > 1, 1, and < 1 indicate a favorable dose reduction, no dose reduction, and a negative dose reduction, respectively. **(C,E)** Combination index (CI) values as a function of the effect levels (Fa), where CI values of > 1, 1, and < 1 indicate synergism, an additive effect, and antagonism, respectively. **(F,G,I)** Flow cytometry to determine the percentage of total apoptotic OS cells. **(H,J)** Colony-forming assay for OS cells. **(K)** Schematic representation of the synergistic effects of ERKi and HCQ on OS cells (n = 5 per group; ***p < 0.001 versus NC+ERKi group; **p < 0.01 versus NC group).

effects than ERKi alone on OS cell proliferation (**Figure 6H**). Cell apoptosis was detected using flow cytometry after annexin V-APC/7-AAD (MultiSciences, China) double staining. Both the percentage of early apoptotic properties and the total apoptotic properties were higher in the group with ERKi treatment than in the NC group; the combined HCQ and ERKi treatment showed an accelerated apoptotic effect (**Figures 6F,G,I**).

Combination of ERKi and HCQ Synergistic Inhibition of OS *in vivo*

We used a right tibial tumor model in mice and performed the treatment process shown in **Figure 7A**. The physical photos of these excised tibial tumors and corresponding volume and weight charts (**Figures 7B,D**) indicated that there was a difference in tumor sizes and weights among the three groups; the data showed that the combination of ERKi and HCQ significantly inhibited tumor growth *in vivo*. H&E staining revealed more tumor cell death in the ERKi + HCQ group (**Figure 7E**) compared to the other groups. Immunohistochemical quantitative analysis confirmed that the expression level of LC3B was higher in the ERKi group than in the NC group, indicating that ERKi promoted autophagic flux of OS *in vivo*. Furthermore, the expression levels of C-MYC were downregulated in the ERKi group compared to the NC group (**Figure 7C**). Moreover, as shown in **Figure 7F**, H&E staining indicated that no histological damage had been observed in multiple organs in the ERKi group and ERKi + HCQ group.

DISCUSSION

ERK is a key downstream protein in the RAS-RAF-MEK-ERK signaling pathway, and its overactivation has been demonstrated in various tumors (Asati et al., 2016). At present, antitumor drugs targeting this pathway, including BRAF inhibitors (vemurafenib, sorafenib, and dabrafenib) as well as MEK inhibitors (such as selumetinib, trametinib, and cobimetinib), have been marketed and have shown to be efficacious in clinical trials. However, it is inevitable that the tumor cells will acquire drug resistance during treatment with this kind of drug. ERK signaling pathways are associated with a variety of substrates such as kinases and transcription factors, and once drug-resistant mutations occur in cells, it causes cell death. Thus, acquired mutations of ERK1/2 are almost absent in tumor cells. Therefore, ERK-targeting drugs are undoubtedly a promising anticancer agent. Previous studies have shown that innate resistance to MEK or RAF inhibitors was reactivated through ERK1/2 in tumor cells (Kidger et al., 2018). Based on the abovementioned studies, compared with RAF inhibitors and MEK inhibitors, ERKis have antitumor effects and can prevent tumor cells from developing drug resistance to a certain extent; thus, they have a broader application prospect in clinical practice. Although no ERK1/2 inhibitor has been officially approved for use, a number of small molecules of ERKis, including Ulixertinib (Sullivan et al., 2018), KO-947, and MK-8353 (Moschos et al., 2018), are currently in the preclinical development phase.

The relationship between autophagy and tumor is complicated: on the one hand, in human tumors, autophagy genes are frequently silenced or mutated, leading to increased stress and tumor progression and indicating that autophagy plays a tumor-suppressor role (Kung et al., 2011; Mowers et al., 2018); however, on the other hand, given the characteristics of abnormal proliferation of tumor cells, their environment has a higher degree of metabolism and other pressures than normal cell environment, making them more dependent on autophagy for their survival (Srinivasan et al., 2017). Furthermore, research on the regulation of autophagy to combat antitumor drug resistance is expanding and becoming increasingly important. TFEB, as a regulatory tool for lysosomal biogenesis, plays an important role in autophagy. TFEB phosphorylation forms a 14–3–3 docking target, resulting in a deactivated TFEB trapped in the cytoplasm (Xu et al., 2019). When TFEB is activated for nuclear transfer, it transmits information about the lysosomal state to the nucleus to trigger a transcriptional response, thereby stimulating autophagy. Further, TFEB nuclear translocation, in association with MAPK/ERK, has been confirmed in a steatohepatitis model (Kim et al., 2017). We found that acute ERK inhibition increases rather than decreases the autophagic flux in OS cells. We believed that this phenomenon may have occurred because acute ERK inhibition regulates TFEB nuclear translocation in OS (**Figure 2J**). As expected, ERKi exacerbated TFEB nuclear translocation in OS cells (**Figures 1H,I**). The molecular mechanisms underlying autophagy inhibition were extremely complex and involved multiple molecules. To further explore this specific mechanism, we used HCQ, an FDA-approved late-stage autophagy inhibitor used in clinical trials, to block the fusion of autophagosomes with lysosomes to inhibit the late stage of autophagy. As expected, HCQ exacerbated the death of ERKi-treated OS cells. In other words, autophagy was activated by ERKi as a compensatory response to maintain cell homeostasis, which in turn further enhanced the dependence of OS cells on autophagy.

Although tumor cells prefer glycolysis as the main mode of energy production and are more dependent on autophagy to remove metabolic waste (Levy et al., 2017), mitochondria still play an important role in the occurrence and development of tumors. In response to the presence of abundant oxygen, the mitochondrial phosphoric acid oxidation is still active. Furthermore, some enzymes needed for glycolysis are located in the mitochondria (Porporato et al., 2018). Moreover, mutations, alterations, and deletions in mitochondrial DNA have been reported in many cancers (Nikolaev, 2018). Recent studies suggest that mitochondrial dynamics and dysfunction are the links among mitochondrial DNA defects, excessive fission, mitochondrial retrograde signaling, and cancer progression (Srinivasan et al., 2017). Mitochondrial oxidative phosphorylation is not impaired in tumor cells and plays an important role in the malignant progression of tumors. A previous study pointed out that mitochondrial dysfunction and oxidative stress trigger a niche favoring cholangiocellular overgrowth and tumorigenesis (Yuan et al., 2017). Moreover, the

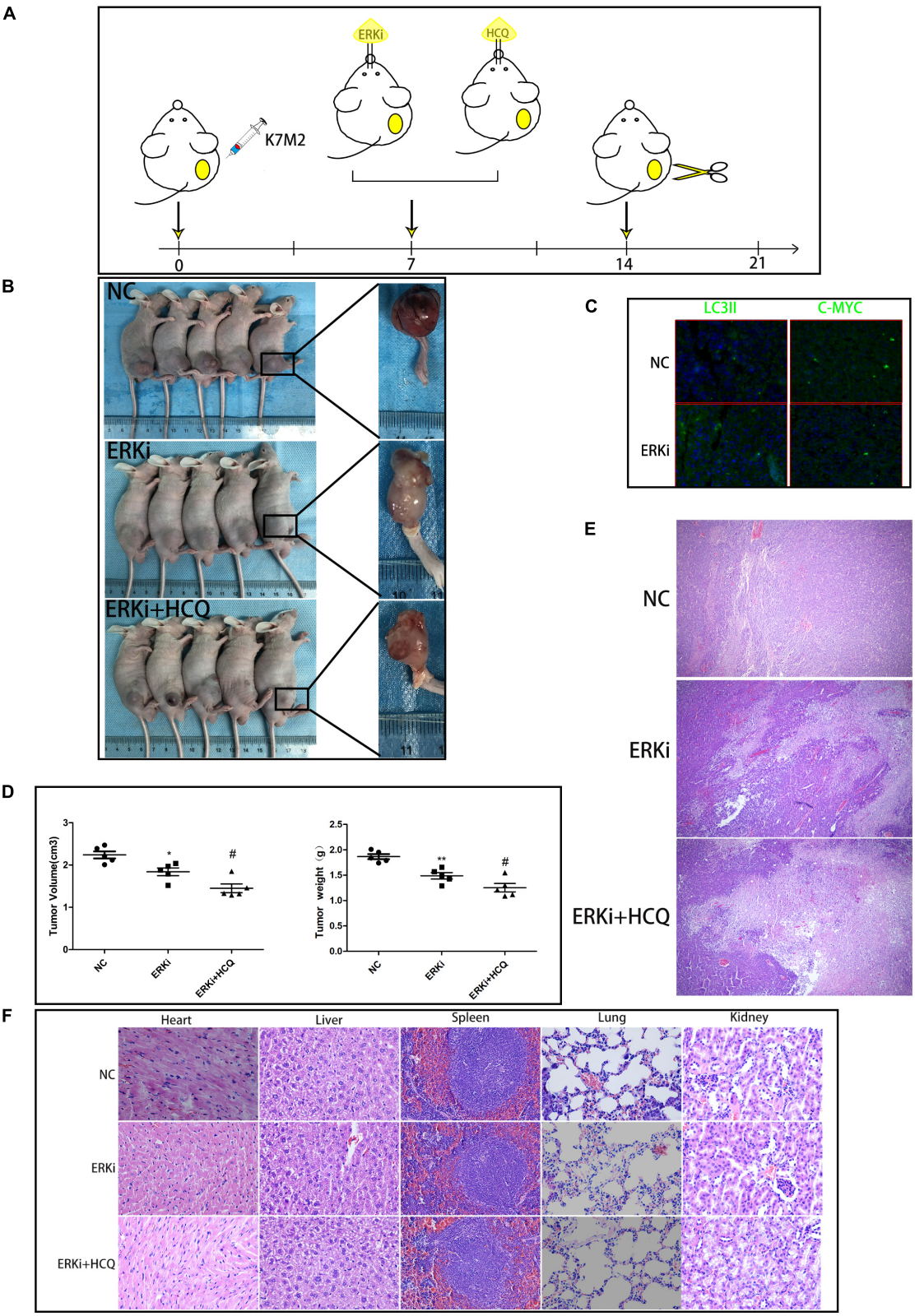


FIGURE 7 | Synergistic inhibitory effect of ERKi and HCQ combined treatment on OS *in vivo*. **(A)** Schematic representation of treatment regimen and surgery. **(B,D)** Tumor formation in different groups. **(C)** Immunofluorescence staining to determine the levels of LC3B and CMYC. **(E)** H&E staining of OS tissue among groups. **(F)** H&E staining of organ tissues of mice administered ERKi or HCQ ($n = 5$ per group; $^{**}p < 0.01$ versus NC group; $^{*}p < 0.05$ versus NC group; $^{\#}p < 0.05$ versus ERKi group).

regulation of ROS plays a very important role in the treatment of cancer. Therefore, some chemoradiotherapy drugs interfere with the outcome of tumors by regulating ROS (Sosaa et al., 2013). Our data showed that ERKi treatment inhibited the activity of OS by increasing the expression of ROS and damaging the antioxidant system. Additionally, ERK induces mitochondrial translocation of phosphoglycerate kinase1 (PGK1) to regulate cancer metabolism and tumorigenesis (Li et al., 2016). Our results showed that in the early stages of ERKi treatment in OS cells, the mitochondria showed a compensatory state, including the increased number and increased expression of fission factors. Later, mitochondrial apoptosis seems to have occurred. This may be the case in the ERKi-treated OS cells: glycolysis activity was suppressed, causing an intake energy disorder in OS cells; thus, there were compensatory increases in mitochondrial activity (**Figure 6K**). However, such compensation is impossible following ERKi-treatment, which may induce mitochondrial apoptotic-related gene expression in OS cells.

CONCLUSION

In this study, we found that inhibition of ERK signaling via pharmacological methods could lead to enhanced anti-OS activity of the autophagy inhibitor. Mechanistically, ERK inhibition directly regulated the nuclear translocation of TFEB to increase autophagy flux and promote mitochondrial apoptosis through the ROS/mitochondria pathway and indirectly increased the dependence of OS cells on autophagy. In addition, we found changes in the mitochondrial dynamics in OS cells during the early stages of ERKi treatment. This may be a manifestation of mitochondrial compensatory tumor promotion, caused by the restraining of aerobic glycolysis by ERKi and the subsequent OS energy-intake disorder.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Research Ethics Committee of Zhejiang University.

AUTHOR CONTRIBUTIONS

YQ conceived and designed the research. MZ and YB performed the experiments. CX and YQ analyzed the data and interpreted results of the experiments. JM, WZ, and HS drafted and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by grants from the National Science Foundation of China (81071259) and the science and technology innovation activity plan of college students in Zhejiang Province (2020R401216).

ACKNOWLEDGMENTS

Thanks for the technical support by the Core Facilities, Zhejiang University School of Medicine.

REFERENCES

Aloia, A., Müllhaupt, D., Chabbert, C. D., Eberhart, T., Flückiger-Mangual, S., Vukolic, A., et al. (2019). A fatty acid oxidation-dependent metabolic shift regulates the adaptation of BRAF-mutated Melanoma to MAPK inhibitors. *Clin. Cancer Res.* 25, 6852–6867. doi: 10.1158/1078-0432.ccr-19-0253

Asati, V., Mahapatra, D. K., and Bharti, S. K. (2016). PI3K/Akt/mTOR and Ras/Raf/MEK/ERK signaling pathways inhibitors as anticancer agents: structural and pharmacological perspectives. *Eur. J. Med. Chem.* 109, 314–341. doi: 10.1016/j.ejmech.2016.01.012

Chao, X., Wang, S., Zhao, K., Li, Y., Williams, J. A., Li, T., et al. (2018). Impaired TFEB-mediated lysosome biogenesis and autophagy promote chronic ethanol-induced liver injury and steatosis in mice. *Gastroenterology* 155, 865-879.e12.

Cuadrado, A., and Nebreda, A. R. (2010). Mechanisms and functions of p38 MAPK signalling. *Biochem J.* 429, 403–417. doi: 10.1042/bj20100323

Giang, A. H., Raymond, T., Brookes, P., de Mesy Bentley, K., Schwarz, E., O'Keefe, R., et al. (2013). Mitochondrial dysfunction and permeability transition in osteosarcoma cells showing the Warburg effect. *J. Biol. Chem.* 288, 33303–33311. doi: 10.1074/jbc.m113.507129

Glick, D., Barth, S., and Macleod, K. F. (2010). Autophagy: cellular and molecular mechanisms. *J. Pathol.* 221, 3–12. doi: 10.1002/path.2697

Gorska-Ponikowska, M., Kuban-Jankowska, A., Eisler, S. A., Perricone, U., Lo Bosco, G., Barone, G., et al. (2018). 2-Methoxyestradiol affects mitochondrial biogenesis pathway and succinate dehydrogenase complex flavoprotein subunit a in osteosarcoma cancer cells. *Cancer Genomics Proteomics* 15, 73–89.

Horbay, R., and Bilyy, R. (2016). Mitochondrial dynamics during cell cycling. *Apoptosis* 21, 1327–1335. doi: 10.1007/s10495-016-1295-5

Hsu, C. C., Tseng, L. M., and Lee, H. C. (2016). Role of mitochondrial dysfunction in cancer progression. *Exp. Biol. Med. (Maywood)* 241, 1281–1295. doi: 10.1177/1535370216641787

Huang, S. T., Bi, K. W., Kuo, H. M., Lin, T. K., Liao, P. L., Wang, P. W., et al. (2014). *Phyllanthus urinaria* induces mitochondrial dysfunction in human osteosarcoma 143B cells associated with modulation of mitochondrial fission/fusion proteins. *Mitochondrion* 17, 22–33. doi: 10.1016/j.mito.2014.05.002

Itoh, H., Kadomatsu, T., Tanoue, H., Yugami, M., Miyata, K., Endo, M., et al. (2018). TET2-dependent IL-6 induction mediated by the tumor microenvironment promotes tumor metastasis in osteosarcoma. *Oncogene* 37, 2903–2920. doi: 10.1038/s41388-018-0160-0

Jawhari, S., Ratinaud, M. H., and Verdier, M. (2016). Glioblastoma, hypoxia and autophagy: a survival-prone 'menage-a-trois'. *Cell Death Dis.* 7: e2434. doi: 10.1038/cddis.2016.318

Karvela, M., Baquero, P., Kuntz, E. M., Mukhopadhyay, A., Mitchell, R., Allan, E. K., et al. (2016). ATG7 regulates energy metabolism, differentiation and survival of Philadelphia-chromosome-positive cells. *Autophagy* 12, 936–948. doi: 10.1080/15548627.2016.1162359

Kidger, A. M., Sipthorp, J., and Cook, S. J. (2018). ERK1/2 inhibitors: new weapons to inhibit the RAS-regulated RAF-MEK1/2-ERK1/2 pathway. *Pharmacol. Ther.* 187, 45–60. doi: 10.1016/j.pharmthera.2018.02.007

Kim, E. K., and Choi, E. J. (2010). Pathological roles of MAPK signaling pathways in human diseases. *Biochim. Biophys. Acta* 1802, 396–405. doi: 10.1016/j.bbadis.2009.12.009

Kim, S. H., Kim, G., Han, D. H., Lee, M., Kim, I., Kim, B., et al. (2017). Ezetimibe ameliorates steatohepatitis via AMP activated protein kinase-TFEB-mediated activation of autophagy and NLRP3 inflammasome inhibition. *Autophagy* 13, 1767–1781. doi: 10.1080/15548627.2017.1356977

Kung, C. P., Budina, A., Balaburski, G., Bergenstock, M. K., and Murphy, M. (2011). Autophagy in tumor suppression and cancer therapy. *Crit. Rev. Eukaryot. Gene Expr.* 21, 71–100. doi: 10.1615/critreveukargeneexpr.v21.i1.50

Levy, J. M. M., Towers, C. G., and Thorburn, A. (2017). Targeting autophagy in cancer. *Nat. Rev. Cancer* 17, 528–542.

Li, X., Jiang, Y., Meisenhelder, J., Yang, W., Hawke, D. H., Zheng, Y., et al. (2016). Mitochondria-translocated PGK1 functions as a protein kinase to coordinate glycolysis and the TCA cycle in tumorigenesis. *Mol. Cell* 61, 705–719. doi: 10.1016/j.molcel.2016.02.009

Marchetti, P., Trinh, A., Khamari, R., and Kluza, J. (2018). Melanoma metabolism contributes to the cellular responses to MAPK/ERK pathway inhibitors. *Biochim. Biophys. Acta Gen. Subj.* 1862, 999–1005. doi: 10.1016/j.bbagen.2018.01.018

Martini-Stoica, H., Xu, Y., Ballabio, A., and Zheng, H. (2016). The autophagy-lysosomal pathway in neurodegeneration: a TFEB perspective. *Trends Neurosci.* 39, 221–234. doi: 10.1016/j.tins.2016.02.002

Meschini, S., Condello, M., Calcabrini, A., Marra, M., Formisano, G., Lista, P., et al. (2008). The plant alkaloid voacamine induces apoptosis-independent autophagic cell death on both sensitive and multidrug resistant human osteosarcoma cells. *Autophagy* 4, 1020–1033. doi: 10.4161/auto.6952

Moschos, S. J., Sullivan, R. J., Hwu, W. J., Ramanathan, R. K., Adjei, A. A., Fong, P. C., et al. (2018). Development of MK-8353, an orally administered ERK1/2 inhibitor, in patients with advanced solid tumors. *JCI Insight* 3, 123–135.

Mowers, E. E., Sharifi, M. N., and Macleod, K. F. (2018). Functions of autophagy in the tumor microenvironment and cancer metastasis. *FEBS J.* 285, 1751–1766. doi: 10.1111/febs.14388

Nikolaev, S. I. (2018). Somatic activating KRAS mutations in arteriovenous malformations of the brain. *N. Engl. J. Med.* 378, 250–261.

Pastore, N., Vainshtein, A., Klisch, T. J., Armani, A., Huynh, T., Herz, N. J., et al. (2017). TFE3 regulates whole-body energy metabolism in cooperation with TFEB. *EMBO Mol. Med.* 9, 605–621. doi: 10.15252/emmm.201607204

Porporato, P. E., Filigheddu, N., Pedro, J. M. B., Kroemer, G., and Galluzzi, L. (2018). Mitochondrial metabolism and cancer. *Cell Res.* 28, 265–280.

Ritter, J., and Bielack, S. S. (2010). Osteosarcoma. *Ann. Oncol.* 21(Suppl. 7): vii320-5.

Samatar, A. A., and Poulikakos, P. I. (2014). Targeting RAS-ERK signalling in cancer: promises and challenges. *Nat. Rev. Drug Discov.* 13, 928–942. doi: 10.1038/nrd4281

Santos, E., and Crespo, P. (2018). The RAS-ERK pathway: a route for couples. *Biochemistry* 11:eaav0917. doi: 10.1126/scisignal.aav0917

Sardiello, M. (2016). Transcription factor EB: from master coordinator of lysosomal pathways to candidate therapeutic target in degenerative storage diseases. *Ann. N. Y. Acad. Sci.* 1371, 3–14. doi: 10.1111/nyas.13131

Sosaa, V., Molinéa, T., Somozaa, R., Paciuccib, R., Kondohc, H., and LLeonarta, M. E. (2013). Oxidative stress and cancer: an overview. *Ageing Res. Rev.* 12, 376–390.

Srinivasan, S., Guha, M., Kashina, A., and Avadhani, N. G. (2017). Mitochondrial dysfunction and mitochondrial dynamics-the cancer connection. *Biochim. Biophys. Acta Bioenerg.* 1858, 602–614. doi: 10.1016/j.bbabio.2017.01.004

Sullivan, R. J., Infante, J. R., Janku, F., Wong, D. J. L., Sosman, J. A., Keedy, V., et al. (2018). First-in-class ERK1/2 inhibitor Ulixertinib (BVD-523) in patients with MAPK mutant advanced solid tumors: results of a phase I dose-escalation and expansion study. *Cancer Discov.* 8, 184–195. doi: 10.1158/2159-8290.cd-17-1119

Wagner, E. F., and Nebreda, A. R. (2009). Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat. Rev. Cancer* 9, 537–549. doi: 10.1038/nrc2694

Wei, H., Wei, S., Gan, B., Peng, X., Zou, W., and Guan, J. L. (2011). Suppression of autophagy by FIP200 deletion inhibits mammary tumorigenesis. *Genes Dev.* 25, 1510–1527. doi: 10.1101/gad.2051011

Xu, Y., Ren, J., He, X., Chen, H., Wei, T., and Feng, W. (2019). YWHA/14-3-3 proteins recognize phosphorylated TFEB by a noncanonical mode for controlling TFEB cytoplasmic localization. *Autophagy* 15, 1017–1030. doi: 10.1080/15548627.2019.1569928

Yong, H. Y., Koh, M. S., and Moon, A. (2009). The p38 MAPK inhibitors for the treatment of inflammatory diseases and cancer. *Expert. Opin. Investig. Drugs* 18, 1893–1905. doi: 10.1517/13543780903321490

Yuan, D., Huang, S., Berger, E., Liu, L., Gross, N., Heinzmann, F., et al. (2017). Kupffer cell-derived tnf triggers cholangiocellular tumorigenesis through JNK due to chronic mitochondrial dysfunction and ROS. *Cancer Cell* 31, 771-789.e6.

Zhang, Y. (2019). The roles of circular RNAs in osteosarcoma. *Med. Sci. Monit.* 25, 6378–6382. doi: 10.12659/msm.915559

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Zhang, Bai, Xu, Qi, Meng, Zhang, Su and Yan. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership