ION CHANNEL SIGNALLING IN CANCER: FROM MOLECULAR MECHANISMS TO THERAPEUTICS

EDITED BY: Elena Adinolfi, Lin-Hua Jiang and Sébastien Roger
PUBLISHED IN: Frontiers in Pharmacology and Frontiers in Oncology



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 978-2-88971-124-6
DOI 10.3389/978-2-88971-124-6

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

ION CHANNEL SIGNALLING IN CANCER: FROM MOLECULAR MECHANISMS TO THERAPEUTICS

Topic Editors:
Elena Adinolfi, University of Ferrara, Italy
Lin-Hua Jiang, University of Leeds, United Kingdom
Sébastien Roger, Université de Tours, France

Citation: Adinolfi, E., Jiang, L.-H., Roger, S., eds. (2021). Ion Channel Signalling in Cancer: From Molecular Mechanisms to Therapeutics.
Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-124-6

Table of Contents

05 ***Editorial: Ion Channel Signalling in Cancer: From Molecular Mechanisms to Therapeutics***
Lin-Hua Jiang, Elena Adinolfi and Sébastien Roger

08 ***Ion Channel Regulation by Sex Steroid Hormones and Vitamin D in Cancer: A Potential Opportunity for Cancer Diagnosis and Therapy***
Iván Restrepo-Angulo, Cecilia Bañuelos and Javier Camacho

21 ***A Compartmentalized Reduction in Membrane-Proximal Calmodulin Reduces the Immune Surveillance Capabilities of $CD8^+$ T Cells in Head and Neck Cancer***
Ameet A. Chimote, Vaibhavkumar S. Gawali, Hannah S. Newton, Trisha M. Wise-Draper and Laura Conforti

37 ***hERG K^+ Channels Promote Survival of Irradiated Leukemia Cells***
Daniela Palme, Milan Misovic, Katrin Ganser, Lukas Klumpp, Helmut R. Salih, Daniel Zips and Stephan M. Huber

51 ***Repurposing Kir6/SUR2 Channel Activator Minoxidil to Arrests Growth of Gynecologic Cancers***
Daniela Fukushiro-Lopes, Alexandra D. Hegel, Angela Russo, Vitalyi Senyuk, Margaret Liotta, Gyda C. Beeson, Craig C. Beeson, Joanna Burdette, Ronald K. Potkul and Saverio Gentile

65 ***A Novel Anti-Kv10.1 Nanobody Fused to Single-Chain TRAIL Enhances Apoptosis Induction in Cancer Cells***
Franziska Hartung, Thomas Krüwel, Xiaoyi Shi, Klaus Pfizenmaier, Roland Kontermann, Patrick Chames, Frauke Alves and Luis A. Pardo

76 ***P2X7 in Cancer: From Molecular Mechanisms to Therapeutics***
Romain Lara, Elena Adinolfi, Catherine A. Harwood, Mike Philpott, Julian A. Barden, Francesco Di Virgilio and Shaun McNulty

106 ***K_V11.1 Potassium Channel and the Na^+/H^+ Antiporter NHE1 Modulate Adhesion-Dependent Intracellular pH in Colorectal Cancer Cells***
Jessica Iorio, Claudia Duranti, Tiziano Lottini, Elena Lastraioli, Giacomo Bagni, Andrea Becchetti and Annarosa Arcangeli

117 ***Voltage-Gated K^+/Na^+ Channels and Scorpion Venom Toxins in Cancer***
Alexis Díaz-García and Diego Varela

127 ***Calcium Permeable Channels in Cancer Hallmarks***
Sendoa Tajada and Carlos Villalobos

146 ***The Functional Role of Voltage-Gated Sodium Channel Nav1.5 in Metastatic Breast Cancer***
Qianxuan Luo, Ting Wu, Wenfang Wu, Gong Chen, Xuan Luo, Liping Jiang, Huai Tao, Mingqiang Rong, Shuntong Kang and Meichun Deng

156 ***Evaluation of P2X7 Receptor Function in Tumor Contexts Using rAAV Vector and Nanobodies (AAVnano)***
Mélanie Demeules, Allan Scarpitta, Catalina Abad, Henri Gondé, Romain Hardet, Carolina Pinto-Espinoza, Anna Marei Eichhoff, Waldemar Schäfer, Friedrich Haag, Friedrich Koch-Nolte and Sahil Adriouch

164 ***TRP Channels and Small GTPases Interplay in the Main Hallmarks of Metastatic Cancer***
Giorgia Chinigò, Alessandra Fiorio Pla and Dimitra Gkika

180 ***Interplay Between Ion Channels and the Wnt/β-Catenin Signaling Pathway in Cancers***
Raphael Rapetti-Mauss, Camille Berenguier, Benoit Allegrini and Olivier Soriani

190 ***Inhibitory Effect of Eslicarbazepine Acetate and S-Licarbazepine on $Na_v1.5$ Channels***
Theresa K. Leslie, Lotte Brückner, Sangeeta Chawla and William J. Brackenbury

203 ***Ion Channel Signature in Healthy Pancreas and Pancreatic Ductal Adenocarcinoma***
Julie Schnipper, Isabelle Dhennin-Duthille, Ahmed Ahidouch and Halima Ouadid-Ahidouch

232 ***Ion Channels Orchestrate Pancreatic Ductal Adenocarcinoma Progression and Therapy***
Verena Hofschröer, Karolina Najder, Micol Rugi, Rayhana Bouazzi, Marco Cozzolino, Annarosa Arcangeli, Gyorgy Panyi and Albrecht Schwab

frontiers in Pharmacology

EDITORIAL
published: 03 June 2021
doi: 10.3389/fphar.2021.711593

Editorial: Ion Channel Signalling in Cancer: From Molecular Mechanisms to Therapeutics

Lin-Hua Jiang[1]*, *Elena Adinolfi*[2] and *Sébastien Roger*[3]

[1]School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds, Leeds, United Kingdom, [2]Department of Medical Sciences, University of Ferrara, Ferrara, Italy, [3]EA4245, Transplantation, Immunology and Inflammation, Faculty of Medicine, University of Tours, Tours, France

Keywords: ion channels, cancer, cell death, proliferation, migration, metastasis, resistance to treatment

Editorial on the Research Topic

Ion Channel Signalling in Cancer: From Molecular Mechanisms to Therapeutics

Mammalian cells express a large number of structurally distinct ion channels on the cell surface and also in the membranes of intracellular organelles. They can selectively permeate ion species (thus named Ca^{2+}, Na^{+}, K^{+} or Cl^{-} channels according to their selectivity) or a group of positively or negatively charged ions (non-selective cation or anion channels) (Hille, 2001). Their activity is regulated by diverse physical, biochemical or biological stimuli of the microenvironments and, as such, they initial or control important signaling mechanisms involved in a wide range of cellular processes which are critical for cancer development and progression including cell differentiation, proliferation, migration and apoptosis. It comes no surprise that an alteration in ion channel expression and/or activity is causatively associated with manifestation of cancer hallmarks (Hanahan and Weinberg, 2011), leading to the introduction of oncochannels (Huber, 2013).

This Research Topic collects 16 review and original research articles, which provide idea-provoking insights or new research regarding the role of ion channels in cancer and the concept or efforts of targeting them to treat cancer.

ROLE OF ION CHANNELS IN CANCER CELLS AND HOST-TUMOUR CROSS-TALK

Intracellular Ca^{2+} homeostasis disruption, and aberrant activation of downstream signalling pathways is a well-established cause of cancerous transformation (Gross et al., 2020). Tajada and Villalobos summarized the expression profile of voltage-gated Ca^{2+} channels ($Ca_V1.2$, $Ca_V1.3$, $Ca_V1.4$, $Ca_V2.1$, $Ca_V2.2$, $Ca_V3.1$, $Ca_V3.2$, $Ca_V3.3$), transient receptor potential channels (TRPC1, TRPC3, TRPC4, TRPV4, TRPV6, TRPM6, TRPM7, TRPM8), and Ca^{2+} release activated Ca^{2+} or store-operated Ca^{2+} (CRAC/SOC) channels (ORAI1, ORAI3) in various cancer types. They also discussed the complex and opposing roles of these Ca^{2+} channels in regulating cancer cell proliferation, migration, invasion, and apoptosis resistance. Finally, they discussed the anti-cancer effects of the available Ca_V and CRAC channel modulators, non-steroidal anti-inflammatory drugs and polyamine biosynthesis inhibitors that directly or indirectly modulate the expression and/or activity of the CRAC channels.

Diaz-Garcia and Varela focused on the voltage-gated K^{+} (K_V) and Na^{+} (Na_V) channels in cancer cells. Several K_V channels, including $K_V1.1$, $K_V1.3$, $K_V10.1$ [also known as the human ether-à-go-go (hEAG)], $K_V11.1$ [also known as the human ether-àgo-go related (hERG) or hERG1] and $K_{Ca}1.1$

OPEN ACCESS

Edited and reviewed by:
Diana Conte Camerino, University of Bari Aldo Moro, Italy

***Correspondence:**
Lin-Hua Jiang
l.h.jiang@leeds.ac.uk

Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *18 May 2021*
Accepted: *24 May 2021*
Published: *03 June 2021*

Citation:
Jiang L-H, Adinolfi E and Roger S (2021) Editorial: Ion Channel Signalling in Cancer: From Molecular Mechanisms to Therapeutics. Front. Pharmacol. 12:711593. doi: 10.3389/fphar.2021.711593

(also known as the big-conductance Ca^{2+}-activated K^+ or BK_{Ca}), are highly expressed in many cancer types, and their overexpression is linked with almost all known cancer hallmarks. Overexpression of the Na_V channels in multiple cancer types, either the pore-forming α-subunits or ancillary β-subunits, is also causatively associated with increased cell migration, invasion and metastasis, exemplified by the neonatal variant $Na_V1.5$ ($nNa_V1.5$) in breast and colorectal cancers, $Na_V1.6$ in cervical and colorectal cancers, and $Na_V1.7$ in prostate, lung and gastrointestinal tract cancers. They also highlighted that some scorpion venom toxins that inhibit the K_V ($K_V1.1$, $K_V1.3$ or BK) or Na_V channels ($Na_V1.6$ or $Na_V\beta1$) have been shown to produce anti-cancer effects, and could therefore be considered as valuable anti-cancer tools.

Schnipper et al. and Hofschröer et al. reviewed a variety of Ca^{2+}, K^+, Na^+ and Cl^- channels involved in pancreatic ductal adenocarcinoma (PDAC). These ion channels include TRP channels (TRPC1, TRPV1, TRPV6, TRPM2, TRPM7, TRPM8), ORAI1 CRAC channel, and ATP-gated Ca^{2+}-permeable channel P2X7 receptor; K_V channels ($K_V1.3$, hEAG, hERG), intermediate Ca^{2+}-activated K^+ channel (IK_{Ca}, also known as $K_{Ca}3.1$), two-pore domain K^+ (K_{2P}) channels (TWIK-1, TREK-1, TASK-1); Na_V and acid-sensitive ion (ASIC1, ASIC3) channels; Ca^{2+}-activated Cl^- channel TMEM16A and related proteins, and cystic fibrosis transmembrane regulator (CFTR). Altered expression of many of these channels, some being upregulated and others downregulated, is known to correlate with low overall patient survival. Hofschröer et al. also described the role of ion channels in the cross-talk between cancer, stroma, and immune cells in PDAC. Indeed, in this malignancy, ion channel activity not only affects cancer cell proliferation and invasion but also extracellular matrix remodeling and fibrosis together with tumour-infiltrating immune cell populations.

Restrepo-Angulo et al. examined the regulation of ion channel expression in cancer cells by sex steroid hormones, such as 17-β estradiol and dihydro-testosterone, and steroid pro-hormones like vitamin D and its active metabolite calcitriol. Exposure to 17-β estradiol, dihydro-testosterone or calcitriol regulated, mainly through a transcriptional mechanism, the expression of ion channels in breast (BK_{Ca}, hEAG, nNav1.5, $Ca_V1.2$, ORAI3, TRPV6, CLC-3), cervical (BK_{Ca}, hEAG), endometrial (BK_{Ca}, $Ca_V1.3$) and prostate (TRPC6) cancers, with upregulation in most cases and downregulation in some cases. Ensuing gain or loss of the channel activity in many but not all cases led to increased cell proliferation, migration, invasion and/or tumor progression. The interesting exception is that calcitriol-induced downregulation of hEAG in breast cancer and TRPC6 in prostate cancer attenuated cell proliferation, suggesting calcitriol as a promising co-adjuvant for the treatment of vitamin D receptor-expressing cancers.

Fukushiro-Lopes et al. examined the role of the ATP-sensitive K^+ (K_{ATP}) channel made of inwardly-rectifying Kir6.2 pore-forming subunit and sulphonylurea receptor 2 (SUR2) regulatory subunit in gynecologic cancers. Treatment with minoxidil, an anti-hypertensive drug that activates the Kir6.2/SUR2 channel via binding to SUR2, prevented development and metastasis in xenogeneic murine models of ovarian carcinoma using human ovarian OVCAR-8 cancer cells. In OVCAR-8 cells and also in human endometrial SPEC-2 cancer cells, treatment with minoxidil resulted in inhibition of cell proliferation via attesting cell recycle, and also induction of apoptosis via Ca^{2+} influx-dependent mitochondrial dysfunction, mitochondrial ROS generation and oxidative DNA damage. These findings raise an attractive possibility of repurposing drugs activating the Kir6.2/SUR2 channel as a new or adjuvant therapeutic approach for ovarian carcinoma.

Palme et al. investigated the effect of ionizing radiation on hERG channel expression in human chronic myeloid leukemia (CML) cells and the implication to radiotherapy. Exposure to sub-lethal radiation induced an increase in the K^+ currents that were sensitive by the hERG inhibitor E4301 in CML K562 cells and primary human CML cells. Treatment with E4301 resulted in a greater increase in the steady-state intracellular Ca^{2+} level in irradiated K562 cells than in control cells, but reduced the activation of Ca^{2+}/calmodulin (CaM)-dependent kinase II. Importantly, treatment with E4301 prevented cell arrest and reduced clonogenic survival of irradiated cells, thus conferring radio-resistance.

As mentioned above, P2X7 is expressed in many cancer types, in stromal and immune cells of the tumor microenvironment (TME) as well as in cancer cells. Lara et al. provided a critical and in-depth analysis of the molecular and signaling mechanisms determining the complex and not yet fully understood role of P2X7 in cancer, or the receptor activity reported so far in different oncological conditions. They also discussed the possible strategies for developing anti-cancer drugs targeting P2X7 with either agonists that induce large pore formation to favor cancer cell death, or antagonists that would reduce P2X7-dependent oncogenic transformation. Chimote et al. analyzed the role of the Ca^{2+}-activated K^+ channel $K_{Ca}3.1$ in tumor infiltration by cytotoxic lymphocytes. They demonstrated that $K_{Ca}3.1$ is central in impaired chemotaxis of $CD8^+$ cells toward the TME in head and neck cancer and that its function critically depends on the level of membrane-associated Ca^{2+} sensor CaM that was reduced in circulating lymphocytes of head and neck cancer patients. Interestingly, $K_{Ca}3.1$ activation with 1-EBIO restored the chemotaxis and tumour-eradicating ability of cells and even amplified the anti-inflammatory response by increasing INFγ production, suggesting that $K_{Ca}3.1$ antagonism could be a suitable therapeutic strategy in head and neck cancer.

INTERPLAYS OF ION CHANNELS WITH OTHER SIGNALING MECHANISMS IN CANCER CELLS

Luo et al. provided an overview of the mechanisms by which $nNa_V1.5$ in breast cancer potentiates cell migration, invasion and metastasis, revealed by *in vitro* and *in vivo* studies mainly using MDA-MB-231 metastatic breast cells. One important mechanism is that $nNa_V1.5$ promotes H^+ efflux via allosteric stimulation of the Na^+/H^+ exchanger isoform-1 (NHE-1) that creates an extracellular acidic microenvironment to facilitate proteinases

to degrade ECM, favoring cell migration and invasion. They also discussed various agents that reduced cell migration and invasion via inhibiting the Nav1.5 expression and activity or altering related signaling pathways.

Iorio et al. reported an important role for NHE1 in mediating hERG-dependent regulation of colorectal cancer (CRC) cell migration, using HCT116 and HT29 cells. Growing cells on surface coated with ECM proteins, collagen I or fibronectin, resulted in intracellular alkalinisation, which was prevented by treatment with NHE-1 inhibitor amiloride, NHE-1-specific inhibitor cariporide or hERG inhibitor E4031, as well as with an β1 integrin-blocking antibody. Cell migration on collagen I-coated surface was also attenuated by treatment with cariporide or E4031. Furthermore, exposure to collagen I enhanced protein-protein interactions of β1 integrin, NHE-1 and hERG. These results suggest that β1 integrin-mediated adhesion of CRC cells onto ECM proteins stimulates β1 integrin, hERG and NHE-1 to form a signaling complex that facilitates hERG-dependent stimulation of NHE-1, leading to H^+ efflux and intracellular alkalinisation to drive cell migration.

Rapetti-Mauss et al. presented an overview of the regulation of the Wnt/β-catenin signaling pathway by ion channels in various cancer types. On one hand, several ion channels including KCNQ1, hERG, CFTR and ClC-2, suppress the Wnt pathway. Consistently, reduction in the expression and/or activity of KCNQ1 in CRC and hepatocellular carcinoma or CFTR and ClC-2 in CRC was shown to stimulate the activity of the Wnt pathway and cancer progression, leading to the notion of these channels as tumor suppressors. On the other hand, overexpression of TRPC5 in CRC, TRPV4 in gastric cancer, TRPM4 in prostate cancer, TRPM8 in prostate and breast cancers, P2X7 in osteosarcoma, and ASIC1a in liver cancer promotes the activity of the Wnt pathway, thereby inducing tumor growth, cell proliferation, migration, invasion, metastasis, and/or chemo-resistance.

Chinigò et al. reviewed the literature regarding bidirectional communications between the TRP channels and small GTPases in various cancer types. The TRP channels were shown to modulate the activity of small GTPase and related signaling pathways in a Ca^{2+}-dependent or independent manner and, conversely, the small GTPases regulate the surface expression or gating of TRP channels. Such interplays were documented to occur between TRPC1, TRPC5, TRPC6, TRPV4, TRPM4 or TRPM7 and Rho GTPases in cell migration, between TRPV2 or TRPM2 and Rho GTPases in cell invasion, and between TRPC1, TRPC6, TRPV4 or TRPM8 and Rho/Ras GTPases in tumor vascularization.

TARGETING ONCOCHANNELS FOR DEVELOPING THERAPEUTIC TOOLS

Recent years have witnessed increasing interest in targeting ion channels with new small molecules and repurposing existing drugs for cancer therapy. For example, Fukushiro-Lopes et al. proposed the Kir6/SUR2 channel activator minoxidil for the treatment of gynecologic cancer. In this vein, Leslie et al. examined the inhibitory effects on $nNa_V1.5$ of eslicarbazepine acetate and slicarbazepine, the major circulating active metabolite and also the main enantiomer responsible for anticonvulsant activity are known to inhibit neuronal non-$Na_V1.5$ channels. Both compounds inhibited $nNa_V1.5$ endogenously expressed in MDA-MB-231 cells and $Na_V1.5$ heterologously expressed in HEK293 cells, leading to the proposal of using such agents to treat metastatic breast cancer.

There are ongoing efforts of developing small molecular chemicals and biologics targeting particular ion channels as research tools to better understand their role in cancers and validate the feasibility of such interventions as cancer therapy. Demeules et al. discussed adeno-associated viral vectors producing P2X7-modfying nanobodies termed AAVnano, and demonstrated the advantage of such an AAVnano to intervene P2X7-mediated cell function or tumor growth in mice. Hartung et al. reported a nanobody against hEAG fused to an apoptosis-inducing ligand and showed its effectiveness in inducing apoptosis and inhibiting spheroid growth using hEAG-expressing human prostate DU-145 cancer cells. These studies highlight the potential of nanobodies, particularly the ones combining cancer specificity and cytotoxicity, as a new approach for cancer therapy.

In summary, studies continue to accumulate the evidence supporting a vital role of an alteration in the expression and/or activity of ion channels in both cancer cells and cancer-contacting cells in determining cancer hallmarks. As such, altered ion channel expression or activity has been proposed by many researchers as a cancer hallmark. Targeting onco-channels has become an attractive strategy of developing novel anti-cancer treatment.

AUTHOR CONTRIBUTIONS

All the authors have contributed to the writing and the revision of this Editorial Article, and approved it for publication.

REFERENCES

Gross, S., Mallu, P., Joshi, H., Schultz, B., Go, C., and Soboloff, J. (2020). Ca2+ as a Therapeutic Target in Cancer. *Adv. Cancer Res.* 148, 233–317. doi:10.1016/bs.acr.2020.05.003

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of Cancer: the Next Generation. *Cell* 144, 646–674. doi:10.1016/j.cell.2011.02.013

Hille, B. (2001). *Ion Channels of Excitable Membranes*. 3rd edition. Massachusetts, United States: Sunderland.

Huber, S. M. (2013). Oncochannels. *Cell Calc.* 53, 241–255. doi:10.1016/j.ceca.2013.01.001

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 Jiang, Adinolfi and Roger. 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 Pharmacology

REVIEW
published: 28 February 2020
doi: 10.3389/fphar.2020.00152

Ion Channel Regulation by Sex Steroid Hormones and Vitamin D in Cancer: A Potential Opportunity for Cancer Diagnosis and Therapy

Iván Restrepo-Angulo[1], *Cecilia Bañuelos*[2] *and Javier Camacho*[1]*

[1] Department of Pharmacology, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Mexico City, Mexico, [2] Transdisciplinary Program on Science, Technology and Society, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Mexico City, Mexico

OPEN ACCESS

Edited by:
Sébastien Roger,
Université de Tours,
France

Reviewed by:
Mathieu Gautier,
University of Picardie Jules Verne,
France
V'Yacheslav Lehen'kyi,
Université de Lille,
France

***Correspondence:**
Javier Camacho
fcamacho@cinvestav.mx

Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: 04 December 2019
Accepted: 05 February 2020
Published: 28 February 2020

Citation:
Restrepo-Angulo I, Bañuelos C and Camacho J (2020) Ion Channel Regulation by Sex Steroid Hormones and Vitamin D in Cancer: A Potential Opportunity for Cancer Diagnosis and Therapy. Front. Pharmacol. 11:152. doi: 10.3389/fphar.2020.00152

Many ion channels are involved in tumor development, promoting cancer cell proliferation, migration, invasion, and survival. Accordingly, some of them have been suggested as tumor markers and novel targets for cancer therapy. Some sex steroid hormones (SSH), including estrogens and androgens, favor cancer progression. Meanwhile, other steroid hormones like vitamin D may have anticancer properties. SSH and vitamin D modulate the expression of a number of ion channels in cancer cells from hormone-sensitive tissues, including breast, ovary, prostate, and cervix. Moreover, rapid effects of SSH may be mediated by their direct action on membrane ion channels. Here, we reviewed the SSH and vitamin D regulation of ion channels involved in cancer, and analyzed the potential molecular pathways implicated. In addition, we described the potential clinical use of ion channels in cancer diagnosis and therapy, taking advantage of their regulation by SSH and vitamin D. Since SSH are considered risk factors for different types of cancer, and ion channels play important roles in tumor progression, the regulation of ion channels by SSH and vitamin D may represent a potential opportunity for early cancer diagnosis and therapeutic approaches in SSH and vitamin D sensitive tumors.

Keywords: steroid hormones, ion channels, cancer, cancer therapy, tumor markers

INTRODUCTION

Sex steroid hormones (SSH) have been considered as a risk factor to develop a variety of human cancers, including those from breast, endometrium, ovary, cervix, colon, and prostate (Maxwell et al., 2009). These hormones act as tumor promoters by inducing proliferation and preventing apoptosis of mutated cells, thus, the possibility of additional genetic and/or epigenetic changes is

Abbreviations: 1,25 VD, 1α,25 dihydroxivitamin D; AR, Androgen receptor; Cav, Voltage-gated calcium channel; ERα, Estrogen receptor alpha; ERβ, Estrogen receptor beta; ERK, Extracellular Receptor Kinase; FAK, Focal Adhesion Kinase; GPR30: G protein-coupled receptor 30; HPV, Human papilloma virus; Kca, Calcium-activated potassium channel; Kv, Voltage-gated potassium channel; MAPK, Mitogen Activated Protein Kinase; Nav, Voltage-gated sodium channel; PKA, Protein Kinase A; SH, Steroid hormones; SSH, Sex steroid hormones; TRP, Transient receptor potential; VDR, Vitamin D receptor.

enhanced (Irigaray and Belpomme, 2010). From a classical point of view, SSH interact with their intracellular receptors, which bind to specific regions of genes involved in cell proliferation and/or differentiation (Bohra and Bhateja, 2015).

Cumulative evidence has shown that ion channels have a role in carcinogenesis by participating in pathways that lead cancer cells to proliferate, survive, migrate, and invade (Kunzelmann, 2005; Prevarskaya et al., 2018). Many ion channels favor tumor progression in hormone-sensitive tissues, hence, the immediate arising question is whether SSH may regulate the expression or activity of ion channels involved in cancer. As the reader will find in this review, SSH exert the transcriptional control of some genes encoding for ion channels. Moreover, SSH not only regulate the activity of ion channels through the canonical transcriptional pathway, besides, they can also bind to channel domains and alter their gating properties, resulting in rapid cellular responses.

This review is intended to compile and show evidence about how ion channels are involved in the carcinogenesis process primarily induced by SSH, namely estrogens and androgens. We also include vitamin D in this review because— although it is not an SSH—it is a steroid hormone (SH) displaying anti-proliferative effects, and regulates some ion channels involved in cancer in hormone-sensitive-tissues. Altogether, ion channels have tremendous potential for translational research as biomarkers for cancer diagnosis and prognosis, as well as for improving the treatment of SH-associated tumors.

STEROID HORMONES: AN OVERVIEW

SH regulate a wide variety of developmental and physiological processes from fetal life to adulthood. These hormones are derived from cholesterol; therefore, they have a closely related structure based on the cyclopentanophenantrene (Miller and Auchus, 2011). They are synthesized and secreted by the adrenal cortex, ovaries, testes, and during pregnancy, by the placenta. Considering their function, steroids can be classified into two major classes: A) *corticosteroids*, which are in turn divided into glucocorticoids, like cortisol and cortisone, which regulate glucose metabolism, and mineralocorticoids, such as aldosterone, that helps to regulate the concentration of sodium and potassium ions in blood; and B) *sex steroids*, which are responsible for the maturity of the reproductive structures, among other functions. Three SSH are known: *estrogens (estradiol),* contributing to the development and maintenance of female secondary sex characteristics; *androgens (testosterone),* that participate in the development and maintenance of male secondary sex characteristics; and *progestagens (progesterone)*, involved in regulating the cyclic changes experienced by the endometrium (Chatuphonprasert et al., 2018). Other SH also comprise *neurosteroids* (those synthesized in the brain, like *allopregnolone*) and *vitamin D,* which is an open-ring steroid, considered as a pro-hormone, because it needs to be transformed in the kidney into its active metabolite, namely 1α,25 dihydroxivitamin D (1,25 VD), also known as calcitriol (Norman, 2008). Throughout the text, SSH will refer to estradiol, progesterone, and testosterone, while SH will include also other hormones like vitamin D.

SH act through receptors located in the cell cytoplasm to control the expression of their target genes. In this transcriptional pathway, the hormone binds to its receptor, inducing a receptor-conformational change. Subsequently, the hormone-receptor complex translocates to the nucleus, where it interacts with specific sequences on the DNA, ultimately resulting in either the up- or down-regulation of the target gene. Transcriptional control of gene expression may take from some hours to a number of days. Examples of these types of receptors are the estrogen receptors alpha and beta (ERα and Erβ), the androgen receptor (AR), and the vitamin D receptor (VDR) (Wilkenfeld et al., 2018). Along with the transcriptional pathway, SH induce rapid, non-transcriptional effects, including blood vessels dilation or tracheal relaxation. It has been proposed that such non-transcriptional actions are produced by hormone binding to either G-protein-coupled membrane receptors or the classical intracellular receptor types embedded into the cell membrane. Examples of these types of receptors are the GPR30 receptor for estrogens (Meyer et al., 2011; Reslan and Khalil, 2012; Wang et al., 2014; Chen et al., 2019), variants of the ERα (Pietras and Márquez-Garbán, 2007; Adlanmerini et al., 2014; Arnal et al., 2017), or the calcium ion channel CatSper, which has a binding domain for prostaglandins and mediates progesterone-induced Ca^{2+} influx in human sperm (Strünker et al., 2011).

Many ion channels regulated by SH in normal cells and tissues have not been yet associated with cancer. However, recognizing such regulation in healthy conditions may provide hints for its potential inclusion in cancer research, as well as to improve our understanding of the participation of SH in different malignancies. Thus, we will review first some examples of the effects of these hormones on ion channels in normal conditions.

STEROID HORMONES REGULATE THE EXPRESSION AND/OR ACTIVITY OF ION CHANNELS IN NORMAL PHYSIOLOGICAL EVENTS

There is a myriad of genes transcriptionally regulated by SH, including a number of ion channels (Kim et al., 2006; Lee and Jeung, 2007; Fraser et al., 2014). Something really fascinating is that SH may also regulate the activity of ion channels by acting on its gating properties, either by inducing the expression of ion channel-interacting proteins, or by direct binding to channels (Majewska et al., 1986; Valverde et al., 1999; Herd et al., 2007; Strünker et al., 2011; Kow and Pfaff, 2016; Morales-Lázaro et al., 2019). The effect of SH on ion channels seems to be a relevant mechanism by which steroids meet many of their physiological functions in both, the cardiovascular and nervous systems, as well as in non-excitable tissues. Next, some examples of these effects in non-cancer cells are discussed.

Cardiovascular System

In general, women at ages before the beginning of menopause are less vulnerable than men to present cardiovascular diseases (Pabbidi et al., 2018). $K_{Ca}1.1$ channels are key elements in the modulation of the vascular smooth muscle tone. One of the most interesting findings associating SSH and ion channels was reported by the end of the last millennium (Valverde et al., 1999), describing that 17 β-estradiol directly binds to the β-regulatory subunit of the KCa1.1 channel, leading to channel activation. More recently, Granados et al. (2019), reported that the estradiol-binding site was located in residues present in the second transmembrane domain of the channel β1 subunit, and that the increase in the open probability of the channel induced by 17 β-estradiol (10 μM) was associated to the voltage-sensor stabilization. These observations suggest a rapid and direct mechanism by which estrogens regulate vascular smooth muscle relaxation, protecting women from cardiovascular diseases in some hormonal conditions. Estrogens also increase the expression of the β1 subunit of this channel in the uterine arterial smooth muscle cells during pregnancy. This increase, in turn, enhances the calcium-dependent opening of channels, resulting in artery dilation (Sakamoto and Kurokawa, 2019).

In cardiomyocytes, progesterone enhances the cardiac slow delayed rectifier potassium current (Iks), and suppresses the L-type calcium current through a non-transcriptional pathway that involves sequential activation of c-Src, PI3-kinase, Akt, eNOS, and nitric oxide (NO) (Kurokawa et al., 2016). Likewise, testosterone promotes Iks current and blocks L-type calcium current in guinea pig cardiac muscle cells (Sakamoto and Kurokawa, 2019). These currents shape the repolarization phase of the cardiac action potential. Therefore, by acting on the L-type (Cav1.x) Ca^{+2} channels and the delayed rectifier K^+ channels, SSH may be partly responsible for the differences in the cardiac electrical activity observed between women and men. However, the role of estradiol in modulating the electrical activity of the heart is not completely understood. Chronic administration of estradiol down-regulates the mRNA levels of the voltage-gated potassium channels Kv1.5 and Kv7.1 in the ventricle of ovarectomized rabbits (Drici et al., 1996). Additionally, estradiol inhibits the rapid delayed rectifier potassium current (Ikr), and increases the duration of the cardiac action potential, as well as the QT interval at physiological concentrations. At higher concentrations, estradiol inhibits Ikr and enhances Iks currents by non-transcriptional pathways (Kurokawa et al., 2016). Moreover, Anneken et al. (2016), showed that estradiol enhances the interaction between chaperone proteins and the subunits of the inward rectifying potassium channel Kv11.1 (KCNH2, ether-à-go-go related gene, ERG), leading to an increased trafficking of the channel towards the cell membrane. This channel is a key element for generating Ikr currents. Estradiol interacts with Kv11.1 channels through their phenylalanine residue 656 (Phe^{656}) at physiological concentrations, and enhances the effect of the selective Kv11.1 channel blocker E-4031. This effect was ablated in aromatase-null mice (Kurokawa et al., 2015), which may explain why some drugs induce long QT in women (Darpo et al., 2014). Some drugs used in breast cancer chemotherapy target either ERα or estrogen synthesis. This hormone-therapy may have either vascular side-effects or even increase the risk of endometrial cancer (for instance in the case of tamoxifen) (Heery et al., 2018). Thus, the effect of estradiol on vascular and cancer-associated ion channels like Kv11.1 or $K_{Ca}1.1$ should be taken into consideration when breast cancer patients receive hormone-therapy.

Neuro-Protection and Neuronal Firing

Increasing evidence suggests that ion channels participate in the protective role of steroids in the central nervous system. For instance, 17-β estradiol significantly prevents glutamate-induced apoptosis in ventro-spinal motoneurons, and in the neuroblastoma hybrid cell line VSC4.1. Estradiol exerts this effect by decreasing intracellular concentrations of Ca^{+2} and caspase activity. Interestingly, the Cav1.x calcium channel blocker nifedipine also achieves a neuro-protective level, seemingly to that observed in cells treated with 17-β estradiol. In addition, 17-β estradiol also inhibited the pro-apoptotic effect induced by the Ca^{+2} channel agonist FPL-64176. These results suggest that 17-β estradiol might prevent apoptosis in neurons by reducing the calcium influx through Cav1.x calcium channels (Sribnick et al., 2009). Sánchez et al. (2014), showed that 17-β estradiol significantly reduced calcium currents flowing through Cav1.x channels in rat cultured cortical neurons, which were also inhibited by the neuro-protective insulin-like growth factor 1 (IGF-1). Conversely, another report showed that 17-β estradiol at picomolar concentrations binds to the dihydropyridine site in the Cav1.x calcium channels, enhancing calcium currents (Vega-Vela et al., 2017). Therefore, the molecular mechanisms involved in Cav1.x channel regulation by estradiol require further investigation. Progesterone also has a protective effect on striatal neurons in a glutamate-induced apoptosis model. Interestingly, progesterone exerts its anti-apoptotic effect by blocking the Cav1.x calcium channel. Such blockade occurs at higher concentrations than those required to activate the progestin receptor, suggesting that the progesterone receptor is likely not necessary for the observed effect of progesterone. Moreover, dose-response experiments showed that at 100 μM, progesterone completely abolished whole-cell calcium currents, indicating that this hormone may affect other types of calcium channels (Luoma et al., 2012).

Estradiol is also involved in modulating the excitability of neurons in different brain nucleus. Zhang et al. (2015) showed that the pore-forming subunit of the voltage gated sodium channel Nav1.1α, and its auxiliary β2-subunit, were two to three fold up-regulated by estradiol (subcutaneous injection of 0.25 μg 17-estradiol benzoate) in neurons of the anteroventral/periventricular preoptic nucleus. This channel plays an important role in maintaining persistent sodium currents, providing these cells with a spontaneous and repetitive burst firing. This firing is required for the high-frequency-stimulated release of kisspeptin, which in turn promotes the activity of neurons expressing the gonadotropin-releasing hormone. Since

some neurotransmitters favor the proliferation, migration and invasion of cancer cells (Jiang et al., 2020), the regulation of neuronal firing by SSH *via* ion channels may provide hints of novel targets for cancer therapy.

Non-Excitable Tissues

A number of interesting reports about the regulation of the activity and expression of ion channels by steroids hormones in non-excitable tissues have been published. Aldosterone induces the expression of the epithelial sodium channel (ENaC) in the colon and kidney. This channel has a pivotal role in sodium re-absorption, which influences the volume of body fluids and blood pressure (Snyder, 2002). Aldosterone also regulates ENaC channels through non-transcriptional mechanisms, by promoting channel trafficking from intracellular compartments to the plasma membrane (Palmer and Frindt, 2016). Estrogens modulate the activity of ENaC channels in the lung epithelium. The open probability of the ENaC channels and its number increased in the rat-alveolar cell line L2 after estradiol treatment. Furthermore, a comparison of lung homogenates from female rats in pro-estrous and di-estrous phases revealed that the highest number of ENaC channels expressed in the cell membrane parallels the highest level of circulating estradiol (Matalon et al., 2015).

Head et al. (1999) observed that progesterone rapidly activates an inward current and increases the intracellular calcium concentration in thymulin-secreting epithelial cells from rat thymus. Because of the rapid response to progesterone, such effect had likely been mediated by a non-transcriptional pathway requiring the activation of a second messenger. Progesterone also enhances the activity of the sperm-specific calcium channel CatSper, by binding the enzyme ABHD2, which promotes the removal of the CatSper inhibitor 2-arachinodoylglycerol (2-AG) (Mannowetz et al., 2017).

Pregnancy is a typical example of a normal physiological hyper-estrogenic condition, thus, it is expected that channels regulated by SSH participate during this process. Accordingly, Kv10.1 (KCNH1, ether-à-go-go-1, EAG1) potassium channel mRNA levels were up-regulated by estradiol in primary cultures from human placental trophoblasts, and human vascular endothelial cells (HUVEC cell line) (Díaz et al., 2009). This effect was prevented by an ERα antagonist. Moreover, potassium currents resembling Kv10.1 channel activity were enhanced by estradiol treatment in syncitium-trophoblasts. Kv10.1 channels are also up-regulated by estradiol in the cervix during pregnancy. While Kv10.1 protein expression in cervical cells was observed in only 26% of non-pregnant women, it was found in 100% of pregnant patients, independently of the trimester of pregnancy or human papillomavirus (HPV) infection (Ramírez et al., 2013). Nevertheless, the precise role of Kv10.1 channels in the placental and cervical tissue remains to be elucidated. The hormonal regulation of ion channels in normal non-cancerous tissues may provide clues to find novel early tumor markers. Following, we will review the regulation of ion channels by SSH and calcitriol in diverse cancer models.

SEX STEROID HORMONES AND VITAMIN D CONTROL THE EXPRESSION AND ACTIVITY OF ION CHANNELS IN CANCER

The regulation of ion channels by estradiol, progesterone, androgens, and calcitriol in different types of cancer has been reported, mainly at the gene and protein expression level, as well as channel activity.

Estrogenic Regulation of Ion Channels

Potassium Channels

SSH modulate the expression and activity of ion channels in cancerous epithelial cells. Díaz et al. (2009) showed that 17-β estradiol, at very low concentrations (10^{-10} M), significantly up-regulated the expression of Kv10.1 channels in the cervical cancer cell line HeLa transfected with ERα. Interestingly, no significative increase in Kv10.1 mRNA levels was observed in HeLa cells lacking ERα. Carlson et al. (2013) pointed out that the effect of 17-β estradiol on Kv10.1 occurs at transcriptional level, since no changes in its biophysical properties were seen when channels were exposed to estradiol concentrations up to 50 μM. Likewise, Kv10.1 mRNA and protein levels were increased in a dose-dependent manner by progesterone (10^{-8} M, 48 h) in HeLa cells bearing the progesterone receptor (Ramírez et al., 2013). The expression of this channel and its regulation by estrogens has also been studied in a transgenic mouse model for cervical cancer development (Ramírez et al., 2018). Transgenic mice expressing the E7 oncogene of HPV and treated with estradiol (0.05 mg pellets subcutaneously inserted), for 3 or 6 months, developed cervical dysplasia and cervical cancer, respectively. Moreover, Kv10.1 mRNA and protein expression were significantly increased by estradiol. The highest expression was found in animals with cervical cancer. Since SSH are considered potential co-factors for cervical cancer development, the regulation of Kv10.1 channels by estradiol and progesterone provides a supplementary mechanism by which these hormones may contribute to cervical cancer progression.

KCa1.1 potassium channels are very interesting proteins because they are overexpressed in hormone-sensitive tumors, and have been involved in different functions relevant in cancer, including cell proliferation and migration (Ge et al., 2014). In addition to their overexpression in gliomas (Liu et al., 2002), their expression is enhanced in tumors influenced by hormones, including breast, ovarian, prostate, and cervical cancer (Bloch et al., 2007; Oeggerli et al., 2012; Ramírez et al., 2018). Extracellular perfusion of 17-β estradiol increased the current flowing through the Maxi-K channels in MCF-7 epithelial breast cancer cells. This effect was not abrogated by treating the cells with the ERα antagonist ICI-182, and it was not necessary for 17-β estradiol to diffuse into the cell to exert its effect on channels (Coiret et al., 2005). The authors concluded that the opening of KCa1.1 channels by 17-β estradiol may represent an alternative pathway by which this hormone induces cell proliferation of epithelial breast cancer cells. In accordance, the inhibition of channel expression or

activity with specific antisense probes or blockers reduced the hormone-induced activation of the channel, as well as cell growth, in breast and prostate cancer cells (Bloch et al., 2007; Oeggerli et al., 2012). This channel was also studied in the HPV-transgenic mice above mentioned. Similarly to Kv10.1 channels, KCa1.1 mRNA and protein expression was significantly increased by estradiol, and the highest expression was found in animals with cervical cancer (Ramírez et al., 2018). Interestingly, multiple estrogen-responsive sequences have been found in the gene promoter of KCa1.1 channels (Kundu et al., 2007). 17-β estradiol also regulates the expression of KCa1.1 channels in endometrial cancer cells. Wang et al. (2018) observed that Ishikawa cells (a human ERα positive endometrial adenocarcinoma cell line) treated with 17-β estradiol (1 nM) presented an increased expression of the KCa1.1 channel protein, as well as phosphorylated ERK (p-ERK) and MEK (p-MEK). Remarkably, by knocking-down the expression of the KCa1.1 channel, the protein levels of p-ERK and p-MEK were also down-regulated. KCa1.1 knockdown also resulted in decreased proliferation, migration, and invasion of Ishikawa cells. These results indicate that KCa1.1 channels could be essential molecules mediating the effects of 17-β estradiol on endometrial cancer Ishikawa cells.

Calcium Channels

Different types of calcium channels have a crucial role in the carcinogenesis process induced by 17-β estradiol. Motiani et al. (2010) showed that ERα-positive breast cancer cell lines (MCF-7, BT474, ZR751, T47D, and HCC1500) display higher protein expression of the store-operated calcium channel Orai3, compared to ERα-negative breast cancer cells. The store-operated calcium entry in ERα-positive breast cancer cells was mediated by Orai3 channels, then, it was proposed that 17-β estradiol induces the expression of these channels in ER positive breast cancer cells. Later, the same group (Motiani et al., 2013) reported that ERα knockdown decreased Orai3 mRNA and protein expression. Additionally, it also reduced store-operated calcium entry, inhibiting the phosphorylation of ERK 1/2 and FAK, and preventing the activation of the transcription factor NFAT1. Orai3 knockdown also decreased the anchorage-independent growth and matrigel invasion of MCF-7 cells, as well as tumorigenesis in mice treated with estradiol (0.72 mg/pellet subcutaneously implanted) (Motiani et al., 2013).

The melastatin transient receptor potential 8 (TRPM8) calcium channel is highly expressed in MCF-7 cells. Interestingly, this channel is significantly down-regulated after culturing cells in estrogen-free medium for 48 to 72 h. However, TRPM8 expression levels can be restored by adding 17-β estradiol (10 nM). ERα knockdown also depressed the levels of TRPM8 mRNA. Furthermore, breast cancer clinical samples display increased expression of TRPM8 channels associated with ERα tumor status (Chodon et al., 2010).

Hao et al. (2015) reported that 17-β estradiol regulates the expression of the subunits Cav1.3—Cav1.4 of the L-type voltage-gated calcium channels. Estradiol increased the expression of Cav1.3 in endometrial cancer cells in a dose- and time-dependent manner. Interestingly, the cell-impermeable protein-conjugated 17-β estradiol (100 nM) also elicited a rapid increase of Cav1.3 expression, which peaked at 30 min. The expression of the Cav1.3 channel was inhibited by pertussin toxin (200 ng/ml), which is a well-known inhibitor of G-coupled protein receptors. A small interfering RNA targeting the GPR30, also inhibited the effect of estradiol-induced up-regulation of the Cav1.3 subunit, and significantly reduced the phosphorylation of ERK ½ and CREB. Moreover, Cav1.3 knockdown markedly impaired estrogen-stimulated calcium influx, cell proliferation, and migration of endometrial cancer cells. Altogether, these data suggest that Cav1.3 is an important component of the molecular setting activated during the carcinogenesis process induced by 17-β estradiol in endometrial cells (Hao et al., 2015).

TRPV6 channels are overexpressed in mammary adenocarcinoma tissue, and play a key role in calcium homeostasis (Zhuang et al., 2002). In accordance, their specific knockdown with siRNA decreased calcium influx by 44% compared to control siRNA. Moreover, cells with TRPV6-targeted knockdown showed less viable cells at 24 and 48 h (35 and 40%, respectively), as well as an increased number of cells in G1 phase at 24 h (Peters et al., 2012). Bolanz et al. (2008) reported that 17-β estradiol (10 nM) up-regulated the expression of TRPV6 in T-47D breast cancer cells, in a time-dependent manner from 24 h of exposure. After 72 h of treatment, TRPV6 mRNA levels increased 69% compared to the basal level. Altogether, these results strongly suggest that TRPV6 channels facilitate the calcium influx required for breast cancer cells to proliferate and survive, and that these channels are part of the molecular mechanism of the 17-β estradiol-induced proliferation in breast cancer cells.

Sodium Channels

Voltage-gated sodium channels are up-regulated in breast cancer. It has been proposed that they potentiate cell motility, endocytosis, and invasion (Mao et al., 2019). These channels are overexpressed in different types of cancers including hormone-sensitive tumors like those from prostate, cervix, and breast. Channel activity and/or expression is even more enhanced in metastatic cells, and channel inhibitors decrease their invasive properties (Diaz et al., 2007; Mao et al., 2019). In the case of breast cancer, the expression of the neonatal spliced form of the Nav1.5 channel has been strongly associated with high metastatic potential *in vitro*, and breast cancer progression *in vivo* (Fraser et al., 2005). Interestingly, there is an inverse correlation between the expression of Nav1.5 and the expression of ERα in breast cancer cells. The metastatic breast cancer cell line MDA- MB-231 lacks ERα and expresses Nav1.5. Conversely, the weak non-metastatic cell line MCF-7 cells expresses ERα, but shows very low expression levels of Nav1.5 (Fraser et al., 2014; Mohammed et al., 2016). Accordingly, MDA-MB-231 cells stably transfected with the ERα, show a significant decrease in the expression and activity of Nav1.5 channels. The channel expression and function were restored after treating cells with the ERα antagonist ICI-182 780 (1 μM) for more than 48 h (Fraser et al., 2014). In this case, the GPR30 acted in opposition to ERα, although its action

seems to involve a non-transcriptional pathway. Extracellular application of 17-β estradiol (10 nM) to MDA-MB-231 cells for a short time (10 s), increased Nav1.5 currents *via* GPR30-dependent activation of PKA, leading to a reduction of cell adhesiveness (Fraser et al., 2010). These results suggest that estradiol might have either, pro- or anti-metastatic effects, depending on the estrogen receptor found in breast cancer cells. Further investigation is needed to elucidate how these pathways may function in patients.

Chloride Channels

Chloride channels participate in different processes of neoplastic transformation, including cell proliferation, migration, invasion, and metastasis (Peretti et al., 2015). Evidence provided by Yang et al. (2018) suggests that the expression of the voltage-gated chloride channel CLC-3 is regulated by 17-β estradiol in breast cancer cells. MCF-7 cells treated with 17-β estradiol showed increased chloride currents that were blocked by 5-nitro-2-(3-phenylpropil-amino) benzoic acid (NPPB), and 4-4′ diisothiocyanatostilbene-2, 2′ disulfonic acid (DIDS). Moreover, data point out to a transcriptional control *via* ERα, since CLC-3 currents were detected in MDA-MB-231 transfected with the ERS1 gene coding for ERα. CLC-3 currents disappeared after treating MCF-7 cells with either the ERα antagonist ICI 182, 780 or the selective estrogen receptor modulator (SERM) tamoxifen. Whether CLC-3 channels are involved in the tumorigenic action of 17-β estradiol on breast cells still needs investigation.

Ion Channel Regulation by Androgens

Androgens play a fundamental role in cell growth and survival in androgen-receptor positive (AR+) prostate cancer cell lines, early-stages of prostate cancer, and even in normal prostate. However, aggressive and late-stage prostate cancers become androgen-independent (Feldman and Feldman, 2001; Yee, 2015). Interestingly, the changes in the sensitivity to androgens parallel modifications in the ion channel expression profile of prostate cancer cells. Different studies showed that androgens exert a transcriptional control on the expression of the TRPM8 calcium channel. Interestingly, the trpm8 gene has 10 putative androgen-responsive elements, one located in the promoter region, and the remaining in introns. Single cell RT-PCR and immunohistochemical analysis revealed that TRPM8 is expressed in androgen-sensitive human prostate apical secretory epithelial cancer cells, but its expression is decreased in cells that lost the androgen receptor. Moreover, TRPM8 channels are down-regulated as prostate cancer cells turn into androgen-independent cells (Gkika and Prevarskaya, 2009). In agreement with these results, Asutkhar et al. (2015) showed that testosterone (1 pM) enhanced Ca^{+2} uptake through endogenous TRPM8 in primary human prostate cells. The effect of testosterone was inhibited by pre-incubating cells with the TRPM8 blocker N-(2-aminoethyl)-N-(4-(benzyloxy)-3-methoxybenzyl)thiophene-2-carboxamide hydrochloride, M8-B (1 μM). Interestingly, these authors suggested that the TRPM8 channel might act as a testosterone receptor, since immunoprecipation with anti-DHT/testosterone IgG showed high levels of TRPM8 protein from different cells including the prostate cancer cell lines LNCaP and PC3, the prostate epithelial cell line RWPE-2 and HEK cells stably expressing TRPM8 channels, after being incubated with testosterone (1 μM) for 3 h. ELISA experiments using purified TRPM8 protein revealed a direct interaction of testosterone with TRPM8. The binding of testosterone was decreased by the presence of TRPM 8 agonists such as menthol (50 μM), icilin (10 μM), and M8-B (1 μM). However, Grolez et al. (2019) showed that, testosterone (10 nM) inhibited the transient increase of the intracellular calcium concentration induced by icillin (10 μM) in the presence of the AR. These results suggest that at some concentrations, testosterone inhibits the activity of TRPM8 *via* an AR-dependent mechanism. Moreover, testosterone (10 μM) facilitates the migration of prostate cancer cells by inhibiting TRPM8 channels. This inhibition was produced by the activated AR interacting with the channel present within lipid-raft domains. Grolez et al. (2019) proposed that the differences between their results and those from Asutkhar et al. (2015) might be explained by the differences in the design of the experiments since the former study used lipid bilayers and cells lacking the AR receptor, as well as lower concentrations of testosterone. Thus, the elucidation of the precise molecular mechanism of androgen regulation of TRPM8 channels requires further investigation.

TRPV6 channels are thought to be involved in cell proliferation under physiological and pathological conditions. Schwartz et al. (2006) showed that TRPV6 channels contribute to the increased proliferation rate of HEK-293 cells in a calcium-dependent manner. In prostate cancer cells, TRPV6 channels are also associated with increased cell proliferation, and resistance to apoptotic stimuli (Lehenkyi et al., 2007; Stewart, 2020). Interestingly, in some cases, the expression of TRPV6 seems to be regulated in a ligand-independent manner because the AR-selective agonist dihydrotestosterone (1 nM), or the AR selective antagonist casodex (10 μM, 24 h), showed no significant effects on TRPV6 expression (Lehenkyi et al., 2007) In contrast, in another study, dihydrotestosterone was shown to inhibit the expression of TRPV6, whereas the AR antagonist bicalutamide promoted its expression (Lehenkyi et al., 2012). In accordance with the latter, the expression of TRPV6 channels is significantly high in most androgen-independent lesions (Maly and Hofmann, 2018). Therefore, the molecular mechanism underlying the effect of androgens on TRPV6 channel expression needs to be elucidated. In addition, electrophysiological data are required to elucidate TRPV6 channel activity in malignant tissues, and how its activity is coupled to cancer progression. Androgens also regulate the expression of the voltage-gated calcium channels Cav3.2. In the prostate cancer cell line LNCaP, neuroendocrine differentiation (or trans-differentiation) induced either, by androgen-free serum or cAMP, was accompanied by an increased proportion of cells expressing Cav3.2 channels, characterized by patch-clamp recordings, pharmacological blockers, and small-interfering RNA (Buchanan and McCloskey, 2016). Trans-differentiation

is a contributing factor to the transition of prostate cancer to the androgen- independent phenotype. At this stage, neuroendocrine cells release mitogenic factors, which may lead to cancer progression and poor prognosis. Cav3.2 channels may have an important role in this process by allowing calcium entry, and favoring mitogen release (Antal and Martin-Caraballo, 2019). Dihydrotestosterone (1 nM for 24 to 72 h) also up-regulates the expression of the Cav1.2 (α1C) subunit of the L-type calcium channels in MCF-7 cells. Further investigation is required to assess the impact of this channel in the calcium signaling occurring in breast cancer cells (Marques et al., 2015).

Regulation of Ion Channels by Vitamin D and Calcitriol

Vitamin D and its active metabolite calcitriol regulate the expression of TRPV6 channels in cancer cells. The human colon cancer cell line CaCo-2 treated with calcitriol (10^{-7} M) for 8 h showed an extraordinary 60-fold increase of TRPV6 mRNA levels, an effect that was completely inhibited by actinomycin (Taparia et al., 2006). A similar result was observed also in CaCo-2 cells overexpressing the VDR and treated with the VDR-agonist curcumin (5 X 10^{-5} M) (Bartik et al., 2010). Interestingly, calcitriol and curcumin have anti-proliferative effects on colon cancer cells. Thus, it is intriguing how TRPV6 channels (which contribute to cell proliferation) may participate in the anti-proliferative effect of vitamin D. Besides, in a low-steroid environment, calcitriol promotes cell proliferation in LNCaP cells, by increasing the expression of TRPV6 channels, which augments calcium uptake (Lehenkyi et al., 2011). It is worth mentioning that calcitriol (100 nM) also up-regulates the expression of TRPV6 in T47D breast cancer cells (Bolanz et al., 2008). Recently, Chen et al. (2018) showed that the expression of the calcium channel TRPV5 inversely correlates with the expression of the VDR in renal cancer cells. Knockdown of TRPV5 in these cells inhibited cell proliferation, migration, and invasion induced by VDR knockdown. These results suggest that VDR acts as a tumor suppressor in renal cancer cells, and that this action includes the suppression of TRPV5 channel expression. Therefore, vitamin D regulates calcium influx by modulating the expression of TRPV6 and TRPV5 channels in a tissue-dependent manner. Calcitriol (10^{-7} M, 24 h) also down-regulates the expression of the Kv10.1 channel in breast and cervical cancer cells (Avila et al., 2010; García-Becerra et al., 2010). Since the VDR antagonist ZK 159222 (10^{-5} M) abrogated the effect of calcitriol on Kv10.1 expression, the authors suggested that this event may occur at transcriptional level. Remarkably, the reduced expression of Kv10.1 induced by calcitriol, parallels the decrease of cell proliferation in cervical cancer cells. Thus, it is possible that the anti-proliferative effect of vitamin D on cervical cancer may be associated to the down-regulation of Kv10.1 channels (Avila et al., 2010; García-Becerra et al., 2010).

Table 1 summarizes data from a number of studies concerning the regulation of different ion channels by SSH and calcitriol in different types of cancer, while **Figure 1** depicts the diverse mechanisms by which SH may regulate ion channel expression and/or activity.

STEROID HORMONE REGULATION OF ION CHANNELS AS A POTENTIAL OPPORTUNITY FOR EARLY TUMOR DIAGNOSIS, PROGNOSIS AND CANCER THERAPY

Ion Channels in Early Detection and Prognosis of Steroid-Responsive Cancers

Since SSH induce carcinogenesis in some tissue, it is expected that some genes regulated by these hormones are either expressed or repressed at the initial stages of the disease, gaining value as potential early cancer biomarkers. In accordance with the increased expression of Kv10.1 channels from low-grade dysplasia to cervical cancer in transgenic mice treated with estrogens (0.05 mg pellets subcutaneously inserted) (Ramírez et al., 2018), Kv10.1 protein expression is also increased in human samples from cervical dysplasia and cancer, as well as in patients taking estrogens. Kv10.1 protein expression was detected in 67% of human cervical cytologies from low-grade intraepithelial lesions, and in 92% of samples from high-grade intraepithelial lesions, but only in 27% of normal non-cancerous samples (Ortiz et al., 2011). In tissue biopsies from different grades of cervical intraepithelial neoplasia (CIN 1-3), it was found that the higher the grade of neoplasia, the higher the expression of Kv10.1 protein (Ortiz et al., 2011). In the same work, it was reported that almost 50% of the patients with normal cervical cytologies, but taking estrogens as either, hormone-replacement or contraceptive therapy, were positive for Kv10.1 protein expression, versus 27% of non-users. These data yielded a statistically significant association between the use of estrogens and Kv10.1 expression. In addition, the presence of the Kv10.1 transcript has been reported in the "tumor free" mammary gland surrounding the breast carcinoma tissue (Hemmerlein et al., 2006). Thus, this channel has been also proposed as an early marker for breast cancer. Besides, Kv10.1 immuno-intensity increases with the grade of breast cancer and results higher in triple-negative samples which belong to the most aggressive phenotype (Liu et al., 2015; Li et al., 2017); however, the potential association between Kv10.1 and ERα expression in breast cancer needs further investigation. Likewise, KCa 1. 1 channels have also been proposed as potential early cervical cancer markers (Ramírez et al., 2018). Protein expression has not been found in human non-cancerous cervix. In contrast, channel protein expression gradually increased from low-grade to high-grade dysplasia and to carcinoma tissues.

Prostate serum antigen (PSA) has been the gold standard technique for detecting prostate cancer in a non-invasive manner; however, new markers that increase the rate of true positive results are still required. TRPM8 channels are promising prostate cancer biomarkers. Bai et al. (2010) found that the mRNA of TRPM 8 is up-regulated in prostate tumor tissue compared to patient-matched non-tumor tissue, however, it did not correlate with the Gleason score. Interestingly, TRPM8 mRNA can be detected in urine and blood of patients with metastatic disease. Probably, the mRNA of TRPM8 is

TABLE 1 | Sex steroid hormone and calcitriol regulation of ion channels in cancer cells.

Hormone Concentration/dose, time of exposure	Ion channel	Effect	Cell type	Cellular process	Reference
17-β Estradiol					
10-14 – 10-10 M, 48 h	Kv10.1	Upregulation	HeLa transfected with ERS1 gene	N/D	Díaz et al., 2009
0.05 mg, 60 days (pellet)		Upregulation	Cervical tissue of HPV-E7 transgenic mice	Tumor progression	Ramírez et al., 2018
Not determined in the patients		Upregulation	Cervical pap-smears from patients taking estrogens	N/D	Ortiz et al., 2011
0.05 mg, 60 days (pellet)	KCa1.1	Upregulation	Cervical tissue of HPV-E7 transgenic mice	Tumor progression	Ramírez et al., 2018
1 nM, 48 and 72 h	KCa1.1	Upregulation	Endometrial cancer Ishikawa cells	Proliferation, migration and invasion	Wang et al., 2018
10 nM, extracelullar perfusion		Increased currents	MCF-7	N/D	Coiret et al., 2005
100 nM,overnight	Orai3	Upregulation	MCF-7	Calcium influx and anchorage-independent growth	Motiani et al., 2013
10 nM, 24 and 48 h	TRPM8	Induced expression	MCF-7		Chodon et al., 2010
100 nM, (time-dependent)	Cav 1.3	Upregulation	Endometrial cancer cells	Calcium influx, proliferation and migration	Hao et al., 2015
10 nM, extracellular perfusion, 10 s	Nav 1.5	Increased currents via GPR30	MDA-MB-231	Decreased cell adhesion	Fraser et al., 2010
100 nM, gradually activated from 500 – 2000 s	CLC-3	Upregulation	MDA-MB-231 transfected with ERS1 gene	N/D	Yang et al., 2018
Dihydro-testosterone					
1 nM, 24 h to 72 h	Cav 1.2	Upregulation	MCF-7 cells	N/D	Marques et al., 2015
1 μM, 3 h	TRPM8	Upregulation	LNCaP and PC3	N/D	Asuthkar et al., 2015
10 nM, 15 min	TRPM8	Inhibition of Ca+2 currents	PC3		Grolez et al., 2019
Calcitriol					
10-7 M, 8 h	TRPV6	Upregulation	CaCo-2	N/D	Taparia et al., 2006; Bartik et al., 2010
100 nM, 2 - 4 days		Upregulation	LNCaP	Cell proliferation	Lehenkyi et al., 2011
100 nM, 24 h		Upregulation	T47D	N/D	Bolanz et al., 2008
10-7 M, 24 h	Kv10.1	Downregulation	SiHa cells transfected with VDR	Decreased cell proliferation	Ávila et al., 2010
		Downregulation	Breast tumor derived cells	Decreased cell proliferation	García-Becerra et al., 2010

N/D, Not determined.

packaged into the extracellular vesicles released by prostate cancer cells, but at the moment this possibility has not been addressed (Kalra et al., 2012). TRPV6 channels have been proposed as prognostic biomarkers for prostate cancer, as well. Fixemer et al. (2003) found that TRPV6 expression significantly correlated with the Gleason score, pathological stage and extra prostatic extension in prostatectomy specimens from 97 clinically organ-confined tumors. Likewise, lymph node metastasis and androgen-insensitive tumors revealed TRPV6 expression in 63 and 67% of cases, respectively. Moreover, Raphaël et al. (2014) demonstrated that TRPV6 channels may be slightly expressed in benign prostate hyperplasia, but its expression increases in prostate carcinomas. These authors concluded that the translocation of the channel to the plasma membrane *via* the Orai1-mediated Ca^{2+}/Annexin I/S100A pathway is the molecular mechanism responsible for the oncogenic potential of TRPV6 channels in prostate cancer. All these results suggest TRPM8 and TRPV6 channels as potential markers for prostate cancer progression and prognosis.

The expression of KCa 1.1 channels was studied in 263 endometrial tissue samples, including 185 type I endometrial cancer, 40 normal endometrial tissues, and 38 atypical hyperplasia samples. KCa1.1 protein expression was significantly elevated in endometrial adenocarcinoma, compared to normal tissue and atypical endometrial hyperplasia. No differences were found between normal endometrial tissue and atypical endometrial hyperplasia. As expected, phosphorylation of ERK and MEK were also increased in endometrial adenocarcinoma samples, compared to normal and hyperplastic endometrial tissue. Interestingly, increased expression of the KCa 1.1 channel was significantly associated with FIGO stage ≥ II and lymph node metastasis (LNM). Additionally, p-ERK up-regulated expression was significantly associated with FIGO stage ≥ II, cervical stromal involvement, lymphovascular space invasion (LVSI), and LNM. These results suggest that up-regulated expression of KCa1.1 channels and p-ERK is related with poor prognosis in type I endometrial cancer (Wang et al., 2018). In summary, ion channel detection in hormone-sensitive tissues or extracellular fluids may serve as potential early markers of SSH-associated cancers. Finally, the regulation of ion channels by SSH in other hormone-sensitive tissues like adrenal gland and testis requires further investigation.

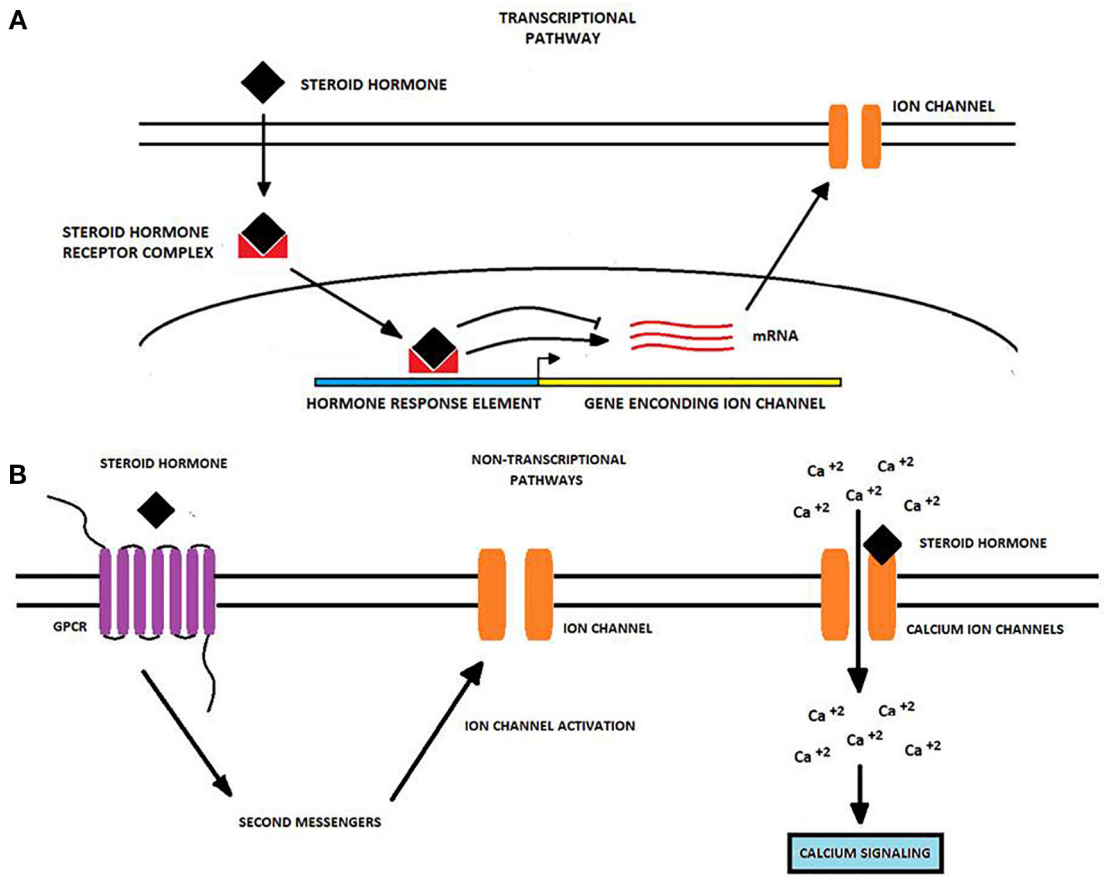


FIGURE 1 | Transcriptional and non-transcriptional regulation of ion channels by steroid hormones in cancer cells. Steroid hormones (SH) regulate the expression and activity of many ion channels *via* two potential molecular pathways. **(A)** The *transcriptional pathway* starts when an SH passively diffuses into the cell and binds an intracellular receptor forming an SH-receptor complex that moves into the nucleus. Here, the complex binds to hormone-response elements to either induce or repress the transcription of genes encoding for ion channels. Upon transcription, ion channel mRNA is translated into the corresponding protein and the ion channel is transported to the cell membrane (Bohra and Bhateja, 2015; Wilkenfeld et al., 2018). **(B)** *Non-transcriptional pathways* primarily involve the binding of the SH to a GPCR triggering second messenger pathways which may activate proteins targeting to ion channels. Alternatively, SH may bind to ion channels like TRPM8 and CatSper modulating its gating properties and enhancing calcium influx (Head et al., 1999; Asuthkar et al., 2015; Grolez et al., 2019). Changes in ion channel expression or activity produced by SH may in turn affect some cancer-associated processes, including cancer cell proliferation, migration, and invasion.

Vitamin D Anti-Proliferative Effects and Ion Channels: A Promising Therapeutic Option for Steroid-Responsive Cancers

Many studies show that calcitriol is a promising co-adjuvant for the treatment of tumors expressing VDR; nevertheless, the use of vitamin D as an adjuvant in cancer therapy is a matter of discussion (Young and Xiong, 2018). The arguments of this discussion are diverse. On one hand, calcitriol has pro-proliferative effects [for instance in prostate cancer cells as above mentioned (Lehenkyi et al., 2011)], and induces hypercalcemia increasing the risk of cardiovascular, gastrointestinal, renal, and neurological- side-effects (Feldenzer and Sarno, 2018). On the other hand, vitamin D has anti-proliferative effects, prevents tumor lesion progression, and improve the quality of cancer patients (Young and Xiong, 2018), although the results are variable between clinical trials. Calcitriol acts as an anti-cancer agent by modulating important regulatory networks that result in the inhibition of cell proliferation, apoptosis and cell differentiation. The transcriptional regulation of some ion channels seems to be a mechanism by which calcitriol has anti-tumoral effects. Garcia-Quiroz et al. (2012) reported that astemizole (an anti-histamine that blocks some members of the EAG channel family) synergistically interacts with calcitriol, at clinically achievable drug concentration, to inhibit cell proliferation of the human breast cancer cells lines T-47D and SUM-229PE. Furthermore, astemizole signicantly increased the growth inhibitory effect of calcitriol by three-fold with a mean inhibitory concentration 20 (IC_{20}) of 1.62 ± 0.75 μM and 1.82 ± 2.41 nM, respectively. When concomitantly used, astemizole (IC_{50} = 2.02 μM) and calcitriol (1 and 10 μM) down-regulate the expression of Ki-67 (a cell proliferation marker) and Kv10.1. Interestingly, astemizole (3 μM) up-regulated the expression of VDR, which might explain the synergic effect. The combined anti-tumoral effect of

astemizole and 1,25 VDR was studied in mice xenografted with TD-47 cells and a primary breast cancer derived-cell culture (MBCDF). Tumor-bearing athymic female mice were treated with oral astemizole (50 mg/kg day) and calcitriol peritoneal injections (0.03 μg/g body weight twice a week) for three weeks. Compared to untreated controls, combined therapy significantly reduced tumor growth. As for cell lines, reduced tumor growth also was accompanied by decreased expression levels of Kv10.1 channels and Ki-67 (García-Quiroz et al., 2014). The synergistic anti-proliferative effect of astemizole and calcitriol has been also observed in hepatocellular carcinoma (Xu et al., 2018), however, the potential involvement of ion channels in this synergy in liver cancer remains elusive.

Calcitriol may also be a useful molecule for the treatment of prostate cancer. Mouse models of full penetrant and slowly evolving prostate tumorigenesis showed that high-calcium diet accelerates the progression of prostate intraepithelial neoplasia by promoting cell proliferation, micro-invasion, tissue inflammation, and the expression of prostate cancer markers. Remarkably, vitamin D prevented the high-calcium diet tumorigenic effect. Data strongly suggested that the antitumor effect of vitamin D and calcitriol included a transcriptional down-regulation of TRPC6 calcium channels. Moreover, the proliferation of PC-3 prostate cancer cells was decreased by silencing the expression of TRPC6 (Bernichtein et al., 2017).

Khatun et al., (2016) showed that calcitriol (1 uM, 72 h) and calcipotriol (1 uM, 72 h), a VDR agonist, inhibited the expression of KCa1.1 channels in MDA-MB-453 breast cancer cells. Then, it was suggested that the transcriptional repression and protein down-regulation of KCa1.1 channels partly contributes to the anti-proliferative effect of the VDR agonist on breast cancer cells.

CONCLUDING REMARKS

A plethora of evidences from cancer cell lines and animal models, as well as human tumor samples, strongly suggests that ion channels are essential components for the association of SSH with cancer. Further translational and clinical research is needed to assess the impact that ion channels blockers, combined with calcitriol or anti-hormone therapy, may have in the treatment of cancer. Nevertheless, by considering that SSH are risk factors for different types of cancer, and ion channels play important roles in tumor progression, the regulation of ion channels by these hormones and calcitriol may serve as an opportunity for early cancer diagnosis and therapeutic approaches in SH-sensitive tumors.

AUTHOR CONTRIBUTIONS

IR-A, CB, and JC contributed conception and design of the review. IR-A wrote the first draft of the manuscript. CB and JC wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

ACKNOWLEDGMENTS

Authors acknowledge all scientific efforts to improve knowledge on cancer and the regulation of ion channels by SH and apologize for research that may have not been cited in this work.

REFERENCES

Adlanmerini, M., Solinhac, R., Abot, A., Fabre, A., Raymond-Letron, I., Guihot, A. L., et al. (2014). Mutation of the palmitoylation site of estrogen receptor a *in vivo* reveals tissue-specific roles for membrane versus nuclear actions. *Proc. Natl. Acad. Sci. U .S. A.* 111 (2), E283–E290. doi: 10.1073/pnas.1322057111

Anneken, L., Baumann, S., Vigneault, P., Biliczki, P., Friedrich, C., Xiao, L., et al. (2016). Estradiol regulates human QT-interval: acceleration of cardiac repolarization by enhanced KCNH2 membrane trafficking. *Eur. Heart J.* 37 (7), 640–650. doi: 10.1093/eurheartj/ehv371

Antal, L., and Martin-Caraballo, M. (2019). T-type calcium channels in cancer. *Cancers* 11 (2), 134. doi: 10.3390/cancers11020134

Arnal, J. F., Lenfant, F., Metivier, R., Flouriot, G., Henrion, D., Adlanmerini, M., et al. (2017). Membrane and nuclear estrogen receptor alpha actions: from tissue specificity to medical implications. *Physiol. Rev.* 97 (3), 1045–1087. doi: 10.1152/physrev.00024.2016

Asuthkar, S., Elustondo, P. A., Demirkhanyan, L., Sun, X., Baskaran, P., Velpula, K. K., et al. (2015). The TRPM8 protein is a testosterone receptor: I. Biochemical evidence for direct TRPM8-testosterone interactions. *J. Biol. Chem.* 290 (5), 2659–2669. doi: 10.1074/jbc.M114.610824

Avila, E., García-Becerra, R., Rodríguez-Rasgado, J. A., Díaz, L., Ordaz-Rosado, D., Zügel, U., et al. (2010). Calcitriol down-regulates human ether à go-go 1 potassium channel expression in cervical cancer cells. *Anticancer Res.* 30, 2667–2672.

Bai, V. U., Murthy, S., Chinnakannu, K., Muhletaler, F., Tejwani, S., Barrack, E. R., et al. (2010). Androgen regulated TRPM8 expression: A potential mRNA marker for metastatic prostate cancer detection in body fluids. *Int. J. Oncol.* 36 (2), 443–450. doi: 10.3892/ijo-00000518

Bartik, L., Whitfield, G. K., Kaczmarska, M., Lowmiller, C. L., Moffet, E. W., Furmick, J. K., et al. (2010). Curcumin: a novel nutritionally derived ligand of the vitamin D receptor with implications for colon cancer chemoprevention. *J. Nutr. Biochem.* 21 (12), 1153–1161. doi: 10.1016/j.jnutbio.2009.09.012

Bernichtein, S., Pigat, N., Delongchamps, N. B., Boutillon, F., Verkarre, V., Camparo, P., et al. (2017). Vitamin D3 prevents calcium-induced progression of early-stage prostate tumors by counteracting TRPC6 and calcium sensing receptor upregulation. *Cancer Res.* 77 (2), 355–365. doi: 10.1158/0008-5472.CAN-16-0687

Bloch, M., Ousingsawat, J., Simon, R., Schraml, P., Gasser, T. C., Mihatsch, M. J., et al. (2007). KCNMA1 gene amplification promotes tumor cell proliferation in human prostate cancer. *Oncogene* 26 (17), 2525–2534. doi: 10.1038/sj.onc.1210036

Bohra, A., and Bhateja, S. (2015). Carcinogenesis and sex hormones: a review. *Endocrinol. Metab. Syndr.* 04 (01), 1–4. doi: 10.4172/2161-1017.1000156

Bolanz, K. A., Hediger, M. A., and Landowski, C. P. (2008). The role of TRPV6 in breast carcinogenesis. *Mol. Cancer Ther.* 7 (2), 271–279. doi: 10.1158/1535-7163.MCT-07-0478

Buchanan, P. J., and McCloskey, K. D. (2016). CaV channels and cancer: canonical functions indicate benefits of repurposed drugs as cancer therapeutics. *Eur. Biophys. J.* 45 (7), 621–633. doi: 10.1007/s00249-016-1144-z

Carlson, A. E., Brelidze, T. I., and Zagotta, W. N. (2013). Flavonoid regulation of EAG1 channels. *J. Gen. Physiol.* 141 (3), 347–358. doi: 10.1085/jgp.201210900

Chatuphonprasert, W., Jarukamjorn, K., and Ellinger, I. (2018). Physiology and pathophysiology of steroid biosynthesis, transport and metabolism in the human placenta. *Front. Pharmacol.* 9 (SEP), 1–29. doi: 10.3389/fphar.2018.01027

Chen, Y. M., Liu, X. Y., Zhang, F. B., Liao, S. F., He, X. Y., Zhuo, D. X., et al. (2018). Vitamin D receptor suppresses proliferation and metastasis in renal cell carcinoma cell lines *via* regulating the expression of the epithelial Ca2+ channel TRPV5. *PloS One* 13 (4), 1–14. doi: 10.1371/journal.pone.0195844

Chen, C., Gong, X., Yang, X., Shang, X., Du, Q., Liao, Q., et al. (2019). The roles of estrogen and estrogen receptors in gastrointestinal disease (Review). *Oncol. Lett.* 18 (6), 5673–5680. doi: 10.3892/ol.2019.10983

Chodon, D., Guilbert, A., Dhennin-Duthille, I., Gautier, M., Telliez, M. S., Sevestre, H., et al. (2010). Estrogen regulation of TRPM8 expression in breast cancer cells. *BMC Cancer* 10, 212. doi: 10.1186/1471-2407-10-212

Coiret, G., Matifat, F., Hague, F., and Ouadid-Ahidouch, H. (2005). 17-β-Estradiol activates maxi-K channels through a non-genomic pathway in human breast cancer cells. *FEBS Lett*. 579 (14), 2995–3000. doi: 10.1016/j.febslet.2005.02.085

Díaz, L., Larrea, F., Avila-Chávez, E., García-Becerra, R., Borja-Cacho, E., Barrera, D., et al. (2009). Estrogens and human papilloma virus oncogenes regulate human Ether-à-go-go-1 potassium channel expression. *Cancer Res.* 69 (8), 3300–3307. doi: 10.1158/0008-5472.CAN-08-2036

Darpo, B., Karnad, D. R., Badilini, F., Florian, J., Garnett, C. E., Kothari, S., et al. (2014). Are women more susceptible than men to drug-induced QT prolongation? Concentration-QTc modelling in a phase 1 study with oral rac-sotalol. *Br. J. Clin. Pharmacol.* 77 (3), 522–531. doi: 10.1111/bcp.12201

Diaz, D., Delgadillo, D., Hernández-Gallegos, E., Ramírez-Domínguez, M., Hinojosa, L. M., Ortiz, C., et al. (2007). Functional expression of voltage-gated sodium channels in primary cultures of human cervical cancer. *J. Cell Physiol.* 210, 469–478. doi: 10.1002/jcp.20871

Drici, M. D., Burklow, T. R., Haridasse, V., Glazer, R. I., and Woosley, R. L. (1996). Sex hormones prolong the QT interval and downregulate potassium channel expression in the rabbit heart. *Circulation* 94 (6), 1471–1474. doi: 10.1161/01.CIR.94.6.1471

Feldenzer, K. L., and Sarno, J. (2018). Hypercalcemia of Malignancy. *J. Adv. Pract. Oncol.* 9 (5), 496–504. doi: 10.6004/jadpro.2018.9.5.4

Feldman, B. J., and Feldman, D. (2001). The development of androgen-independent prostate cancer. *Nat. Rev. Cancer* 1 (1), 34–35. doi: 10.1038/35094009

Fixemer, T., Wissenbach, U., Flockerzi, V., and Bonkhoff, H. (2003). Expression of the Ca^{2+}-selective cation channel TRPV6 in human prostate cancer: a novel prognostic marker for tumor progression. *Oncogene* 22, 7858–7861. doi: 10.1038/sj.onc.1206895

Fraser, S. P., Diss, J. K. J., Chioni, A. M., Mycielska, M. E., Pan, H., Yamaci, R. F., et al. (2005). Voltage-gated sodium channel expression and potentiation of human breast cancer metastasis. *Clin. Cancer Res.* 11 (15), 5381–5389. doi: 10.1158/1078-0432.CCR-05-0327

Fraser, S. P., Ozerlat-Gunduz, I., Onkal, R., Diss, J. K. J., Latchman, D. S., and Djamgoz, M. B. A. (2010). Estrogen and non-genomic upregulation of voltage-gated na+ channel activity in mda-mb-231 human breast cancer cells: role in adhesion. *J. Cell. Physiol.* 224 (2), 527–539. doi: 10.1002/jcp.22154

Fraser, S. P., Ozerlat-Gunduz, I., Brackenbury, W. J., Fitzgerald, E. M., Campbell, T. M., Coombes, R. C., et al. (2014). Regulation of voltage-gated sodium channel expression in cancer: hormones, growth factors and auto-regulation. *Philos. Trans. R. Soc. B: Biol. Sci.* 369. doi: 10.1098/rstb.2013.0105

García-Becerra, R., Díaz, L., Camacho, J., Barrera, D., Ordaz-Rosado, D., Morales, A., et al. (2010). Calcitriol inhibits Ether-à go-go potassium channel expression and cell proliferation in human breast cancer cells. *Exp. Cell Res.* 316 (3), 433–442. doi: 10.1016/j.yexcr.2009.11.008

García-Quiroz, J., García-Becerra, R., Barrera, D., Santos, N., Avila, E., Ordaz-Rosado, D., et al. (2012). Astemizole synergizes calcitriol antiproliferative activity by inhibiting CYP24A1 and upregulating VDR: a novel approach for breast cancer therapy. *PloS One* 7 (9), 1–14. doi: 10.1371/journal.pone.0045063

García-Quiroz, J., García-Becerra, R., Santos-Martínez, N., Barrera, D., Ordaz-Rosado, D., Avila, E., et al. (2014). In vivo dual targeting of the oncogenic ether-à-go-go-1 potassium channel by calcitriol and astemizole results in enhanced antineoplastic effects in breast tumors. *BMC Cancer* 14 (1), 1–10. doi: 10.1186/1471-2407-14-745

Ge, L., Hoa., N. T., Wilson, Z., Arismendi-Morillo, G., Kong, X. T., Tajhya, R. B., et al. (2014). Big Potassium (BK) ion channels in biology, disease and possible targets for cancer immunotherapy. *Int. Immunopharmacol.* 22 (2), 427–443. doi: 10.1016/j.intimp.2014.06.040

Gkika, D., and Prevarskaya, N. (2009). Molecular mechanisms of TRP regulation in tumor growth and metastasis. *Biochim. Biophys. Acta (BBA) - Mol. Cell Res.* 1793 (6), 953–958. doi: 10.1016/J.BBAMCR.2008.11.010

Granados, S. T., Castillo, K., Bravo-Moraga, F., Sepúlveda, R. V., Carrasquel-Ursulaez, W., Rojas, M., et al. (2019). The molecular nature of the 17β-Estradiol binding site in the voltage- and Ca^{2+}-activated K^+ (BK) channel β1 subunit. *Sci. Rep.* 9 (1), 9965. doi: 10.1038/s41598-019-45942-1

Grolez, G. P., Gordiendko, D. V., Clarisse, M., Hammadi, M., Desruelles, E., Fromont, G., et al. (2019). TRPM8-androgen receptor association within lipid rafts promotes prostate cancer cell migration. *Cell Death Dis.* 10 (9), 652. doi: 10.1038/s41419-019-1891-8

Hao, J., Bao, X., Jin, B., Wang, X., Mao, Z., Li, X., et al. (2015). Ca $^{2+}$ channel subunit α 1D promotes proliferation and migration of endometrial cancer cells mediated by 17β-estradiol *via the* G protein-coupled estrogen receptor. *FASEB J.* 29 (7), 2883–2893. doi: 10.1096/fj.14-265603

Head, G. M., Downing, J. E. G., Brucker, C., Mentlein, R., and Kendall, M. D. (1999). Rapid progesterone actions on thymulin-secreting epithelial cells cultured from rat thymus. *NeuroImmunoModulation* 6 (1–2), 31–38. doi: 10.1159/000026361

Heery, M., Corbett, P., and Zelkowitz, R. (2018). Precautions for patients taking tamoxifen. *J. Adv. Pract. Oncol.* 9 (1), 78–83. doi: 10.6004/jadpro.2018.9.1.6

Hemmerlein, B., Weseloh, R. M., Mello de Queiroz, F., Knötgen, H., Sánchez, A., Rubio, M. E., et al. (2006). Overexpression of Eag1 potassium channels in clinical tumors. *Mol. Cancer* 5, 41. doi: 10.1186/1476-4598-5-41

Herd, M. B., Belelli, D., and Lambert, J. J. (2007). Neurosteroid modulation of synaptic and extrasynaptic GABAA receptors. *Pharmacol. Ther.* 116 (1), 20–34. doi: 10.1016/j.pharmthera.2007.03.007

Irigaray, P., and Belpomme, D. (2010). Basic properties and molecular mechanisms of exogenous chemical carcinogens. *Carcinogenesis* 31 (2), 135–148. doi: 10.1093/carcin/bgp252

Jiang, S. H., Hu, L. P., Wang, X., Li, J., and Zhang, Z. G. (2020). Neurotransmitters: emerging targets in cancer. *Oncogene* 39 (3), 503–515. doi: 10.1038/s41388-019-1006-0

Kalra, H., Simpson, R. J., Ji, H., Aikawa, E., Altevogt, P., Askenase, P., et al. (2012). Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. *PloS Biol.* 10 (12), e1001450. doi: 10.1371/journal.pbio.1001450

Khatun, A., Fujimoto, M., Kito, H., Niwa, S., Suzuki, T., and Ohya, S. (2016). Down-regulation of ca2+-activated K+ channel KCa1.1 in human breast cancer MDA-MB-453 cells treated with vitamin D receptor agonists. *Int. J. Mol. Sci.* 17 (12), 2083. doi: 10.3390/ijms17122083

Kim, H. J., Lee, G. S., Ji, Y. K., Choi, K. C., and Jeung, E. B. (2006). Differential expression of uterine calcium transporter 1 and plasma membrane Ca2+ ATPase 1b during rat estrous cycle. *Am. J. Physiol. - Endocrinol. Metab.* 291 (2), 234–241. doi: 10.1152/ajpendo.00434.2005

Kow, L. M., and Pfaff, D. W. (2016). Rapid estrogen actions on ion channels: a survey in search for mechanisms. *Steroids*. 111, 46–53. doi: 10.1016/j.steroids.2016.02.018

Kundu, P., Alioua, A., Stefani, E., and Toro, L. (2007). Regulation of mouse Slo gene expression: multiple promoters, transcription start sites, and genomic action of estrogen. *J. Biol. Chem.* 282 (37), 27478–27492. doi: 10.1074/jbc.M704777200

Kunzelmann, K. (2005). Ion channels and cancer. *J. Membr. Biol.* 205 (3), 159–173. doi: 10.1007/s00232-005-0781-4

Kurokawa, J., Sasano, T., Kodama, M., Li, M., Ebana, Y., Harada, N., et al. (2015). Aromatase knockout mice reveal an impact of estrogen on drug-induced alternation of murine electrocardiography parameters. *J. Toxicol. Sci.* 40 (3), 339–348. doi: 10.2131/jts.40.339

Kurokawa, J., Kodama, M., Clancy, C. E., and Furukawa, T. (2016). Sex hormonal regulation of cardiac ion channels in drug-induced QT syndromes. *Pharmacol. Ther.* 168, 23–28. doi: 10.1016/j.pharmthera.2016.09.004

Lee, G. S., and Jeung, E. B. (2007). Uterine TRPV6 expression during the estrous cycle and pregnancy in a mouse model. *Am. J. Physiol. - Endocrinol. Metab.* 293 (1), 132–138. doi: 10.1152/ajpendo.00666.2006

Lehenkyi, V., Flourakis, M., Skryma, R., and Prevarskaya, N. (2007). TRPV6 channel controls prostate cancer cell proliferation *via* Ca 2+/NFAT-dependent pathways. *Oncogene*. 25 (52), 7380–7385. doi: 10.1038/sj.onc.1210545

Lehenkyi, V., Raphaël, M., Oulidi, A., Flourakis, M., Khalimonchyk, S., Kondratskyi, A., et al. (2011). TRPV6 determines the effect of vitamin D3 on prostate cancer cell growth. *PloS One* 6 (2), e16856. doi: 10.1371/journal.pone.0016856

Lehenkyi, V., Raphaël, M., and Prevarskaya, N. (2012). The role of the TRPV6 channel in cancer. *J. Physiol.* 590 (6), 1369–1376. doi: 10.1113/jphysiol.2011.225862

Li, Z., Zhu, K., Gong, X., Vasilescu, S., Sun, Y., Hong, K., et al. (2017). Inducing Polyclonal Eag1-specific antibodies by vaccination with a linear epitope immunogen and its relation to breast tumorigenesis. *Pathol. Oncol. Res.* 23 (4), 761–767. doi: 10.1007/s12253-016-0158-2

Liu, X., Chang, Y., Reinhart, P. H., Sontheimer, H., and Chang, Y. (2002). Cloning and characterization of glioma BK, a novel BK channel isoform highly expressed in human glioma cells. *J. Neurosci.* 22, 1840–1849. doi: 10.1523/JNEUROSCI.22-05-01840.2002

Liu, G. X., Yu, Y. C., He, X. P., Ren, S. N., Fang, X. D., Liu, F., et al. (2015). Expression of eag1 channel associated with the aggressive clinicopathological features and subtype of breast cancer. *Int. J. Clin. Exp. Pathol.* 8 (11), 15093–15099.

Luoma, J. I., Stern, C. M., and Mermelstein, P. G. (2012). Progesterone inhibition of neuronal calcium signaling underlies aspects of progesterone-mediated neuroprotection. *J. Steroid Biochem. Mol. Biol.* 131 (1–2), 30–36. doi: 10.1016/j.jsbmb.2011.11.002

Majewska, M. D., Harrison, N. L., Schwartz, R. D., Barker, J. L., and Paul, S. M. (1986). Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science.* 232 (4753), 1004–1007. doi: 10.1126/science.2422758

Maly, I., and Hofmann, W. (2018). Calcium and nuclear signaling in prostate cancer. *Int. J. Mol. Sci.* 19 (4), 1237. doi: 10.3390/ijms19041237

Mannowetz, N., Miller, M. R., and Lishko, P. V. (2017). Regulation of the sperm calcium channel CatSper by endogenous steroids and plant triterpenoids. *Proc. Natl. Acad. Sci. U.S.A.* 114 (22), 5743–5748. doi: 10.1073/pnas.1700367114

Mao, W., Zhang, J., Körner, H., Jiang, Y., and Ying, S. (2019). The emerging role of voltage-gated sodium channels in tumor biology. *Front. Oncol.* 9, 124. doi: 10.3389/fonc.2019.00124

Marques, R., Peres, C. G., Vaz, C. V., Gomes, I. M., Figueira, M. I., Cairrão, E., et al. (2015). 5α-Dihydrotestosterone regulates the expression of L-type calcium channels and calcium-binding protein regucalcin in human breast cancer cells with suppression of cell growth. *Med. Oncol.* 32, 228. doi: 10.1007/s12032-015-0676-x

Matalon, S., Bartoszewski, R., and Collawn, J. F. (2015). Role of epithelial sodium channels in the regulation of lung fluid homeostasis. *Am. J. Physiol. - Lung Cell. Mol. Physiol.* 309 (11), L1229–L1238. doi: 10.1152/ajplung.00319.2015

Maxwell, G. L., Jazaeri, A. A., and Rice, L. W. (2009). "Hormones and human malignancies," in *Principles and practice of gynecologic oncology*, *5th Ed*. Eds. R. R. Barakat, M. Markman and M. E. Randall (China: Wolters Kluvers).

Meyer, M. R., Prossnitz, E. R., and Barton, M. (2011). The G protein-coupled estrogen receptor GPER/GPR30 as a regulator of cardiovascular function. *Vasc. Pharmacol.* 55 (1–3), 17–25. doi: 10.1016/J.VPH.2011.06.003

Miller, W. L., and Auchus, R. J. (2011). The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr. Rev.* 32 (1), 81–151. doi: 10.1210/er.2010-0013

Mohammed, F. H., Khajah, M. A., Yang, M., Brackenbury, W. J., and Luqmani, Y. A. (2016). Blockade of voltage-gated sodium channels inhibits invasion of endocrine-resistant breast cancer cells. *Int. J. Oncol.* 48 (1), 73–83. doi: 10.3892/ijo.2015.3239

Morales-Lázaro, S. L., González-Ramírez, R., and Rosenbaum, T. (2019). Molecular interplay between the sigma-1 receptor, steroids, and ion channels. *Front. Pharmacol.* 10 (APR), 1–12. doi: 10.3389/fphar.2019.00419

Motiani, R. K., Abdullaev, I. F., and Trebak, M. (2010). A novel native store-operated calcium channel encoded by Orai3: selective requirement of Orai3 versus Orai1 in estrogen receptor-positive versus estrogen receptor-negative breast cancer cells. *J. Biol. Chem.* 285 (25), 19173–19183. doi: 10.1074/jbc.M110.102582

Motiani, R. K., Zhang, X., Harmon, K. E., Keller, R. S., Matrougui, K., Bennett, J. A., et al. (2013). Orai3 is an estrogen receptor α-regulated Ca2+ channel that promotes tumorigenesis. *FASEB J.* 27 (1), 63–75. doi: 10.1096/fj.12-213801

Norman, A. W. (2008). From vitamin D to hormone D: fundamentals of the vitamin D endocrine system essential for good health. *Am. J. Clin. Nutr.* 88 (2), 491S–499S. doi: 10.1093/ajcn/88.2.491s

Oeggerli, M., Tian, Y., Ruiz, C., Wijker, B., Sauter, G., Obermann, E., et al. (2012). Role of KCNMA1 in breast cancer. *PloS One* 7, e41664. doi: 10.1371/journal.pone.0041664

Ortiz, C. S., Montante-Montes, D., Saqui-Salces, M., Hinojosa, L. M., Gamboa-Dominguez, A., Hernández-Gallegos, E., et al. (2011). Eag1 potassium channels as markers of cervical dysplasia. *Oncol. Rep.* 26 (6), 1377–1383. doi: 10.3892/or.2011.1441

Pabbidi, M. R., Kuppusamy, M., Didion, S. P., Sanapureddy, P., Reed, J. T., and Sontakke, S. P. (2018). Sex differences in the vascular function and related mechanisms: role of 17b-estradiol. *Am. J. Physiol. Heart Circ. Physiol.* 315, H1499–H1518. doi: 10.1152/ajpheart.00194.2018

Palmer, L. G., and Frindt, G. (2016). Regulation of epithelial Na channels by aldosterone. *Kitasato Med. J.* 46, 1–7.

Peretti, M., Angelini, M., Savalli, N., Florio, T., Yuspa, S. H., and Mazzanti, M. (2015). Chloride channels in cancer: focus on chloride intracellular channel 1 and 4 (CLIC1 AND CLIC4) proteins in tumor development and as novel therapeutic targets. *Biochim. Biophys. Acta* 1848 (10), 2523–2531. doi: 10.1016/J.BBAMEM.2014.12.012

Peters, A. A., Simpson, P. T., Bassett, J. J., Lee, J. M., Da Silva, L., Reid, L. E., et al. (2012). Calcium channel TRPV6 as a potential therapeutic target in estrogen receptor-negative breast cancer. *Mol. Cancer Ther.* 11 (10), 2158–2168. doi: 10.1158/1535-7163.MCT-11-0965

Pietras, R. J., and Márquez-Garbán, D. C. (2007). Membrane-associated estrogen receptor signaling pathways in human cancers. *Clin. Cancer Res.* 13 (16), 4672–4676. doi: 10.1158/1078-0432.CCR-07-1373

Prevarskaya, N., Skryma, R., and Shuba, Y. (2018). Ion channels in cancer: Are cancer hallmarks oncochannelopathies? *Physiol. Rev.* 98 (2), 559–621. doi: 10.1152/physrev.00044.2016

Ramírez, A., Hinojosa, L. M., Gonzales, J., d., J., Montante-Montes, D., Martínez-Benítez, B., et al. (2013). KCNH1 potassium channels are expressed in cervical cytologies from pregnant patients and are regulated by progesterone. *Reproduction* 146, 615–623. doi: 10.1530/REP-13-0318

Ramírez, A., Vera, E., Gamboa-Domínguez, A., Lambert, P., Gariglio, P., and Camacho, J. (2018). Calcium-activated potassium channels as potential early markers of human cervical cancer. *Oncol. Lett.* 15 (5), 7249–7254. doi: 10.3892/ol.2018.8187.

Raphaël, M., Lehenkyi, V., Vandenberghe, M., Beck, B., Khalimonchyk, S., Vanden Abeele, F., et al. (2014). TRPV6 calcium channel translocates to the plasma membrane *via* Orai1-mediated mechanism and controls cancer cell survival. *Proc. Natl. Acad. Sci. U .S. A.* 111 (37), E3870–E3879. doi: 10.1073/pnas.1413409111

Reslan, O. M., and Khalil, R. A. (2012). Vascular Effects of Estrogenic Menopausal Hormone Therapy. *Rev. Recent Clin. Trials* 7 (1), 47–70. doi: 10.2174/157488712799363253

Sánchez, J. C., López-zapata, D. F., and Pinzón, O. A. (2014). Effects of 17beta-estradiol and IGF-1 on L-type voltage-activated and stretch-activated calcium currents in cultured rat cortical neurons. *Neuro. Endocrinol. Lett.* 35 (8), 724–732.

Sakamoto, K., and Kurokawa, J. (2019). Involvement of sex hormonal regulation of K+ channels in electrophysiological and contractile functions of muscle tissues. *J. Pharmacol. Sci.* 139 (4), 259–265. doi: 10.1016/j.jphs.2019.02.009

Schwarz, E. C., Wissenbach, U., Niemeyer, B. A., Strauß, B., Philipp, S. E., Flockerzi, V., et al. (2006). TRPV6 potentiates calcium-dependent cell proliferation. *Cell Calcium* 39 (2), 163–173. doi: 10.1016/J.CECA.2005.10.006

Snyder, P. M. (2002). The epithelial Na+ channel: cell surface insertion and retrieval in Na+ homeostasis and hypertension. *Endocr. Rev.* 23 (2), 258–275. doi: 10.1210/edrv.23.2.0458

Sribnick, E. A., Del Re, A. M., Ray, S. K., Woodward, J. J., and Banik, N. L. (2009). Estrogen attenuates glutamate-induced cell death by inhibiting Ca2+ influx through L-type voltage-gated Ca2+ channels. *Brain Res.* 1276, 159–170. doi: 10.1016/J.BRAINRES.2009.04.022

Stewart, J. M. (2020). TRPV6 as a target for cancer therapy. *J. Cancer.* 11 (2), 374–387. doi: 10.7150/jca.31640

Strünker, T., Goodwin, N., Brenker, C., Kashikar, N. D., Weyand, I., Seifert, R., et al. (2011). The CatSper channel mediates progesterone-induced Ca2+ influx in human sperm. *Nature*. 471 (7338), 382–386. doi: 10.1038/nature09769

Taparia, S., Fleet, J. C., Peng, J. B., Wang, X. D., and Wood, R. J. (2006). 1,25-Dihydroxyvitamin D and 25-hydroxyvitamin D - mediated regulation of TRPV6 (a putative epithelial calcium channel) mRNA expression in Caco-2 cells. *Eur. J. Nutr*. 45 (4), 196–204. doi: 10.1007/s00394-005-0586-3

Valverde, M. A., Rojas, P., Amigo, J., Cosmelli, D., Orio, P., Bahamonde, M. I., et al. (1999). Acute activation of Maxi –K channels (hSlo) by estradiol binding to the β subunit. *Science* 285 (5435), 1929–1931. doi: 10.1126/science.285.5435.1929.

Vega-Vela, N. E., Osorio, D., Avila-Rodriguez, M., Gonzalez, J., García-Segura, L. M., Echeverria, V., et al. (2017). L-type calcium channels modulation by estradiol. *Mol. Neurobiol.* 54 (7), 4996–5007. doi: 10.1007/s12035-016-0045-6

Wang, C., Liu, Y., and Cao, J. M. (2014). G protein-coupled receptors: extranuclear mediators for the non-genomic actions of steroids. *Int. J. Mol. Sci.* 15 (9), 15412–15425. doi: 10.3390/ijms150915412

Wang, F., Chen, Q., Huang, G., Guo, X., Li, N., Li, Y., et al. (2018). BKCa participates in E2 inducing endometrial adenocarcinoma by activating MEK/ERK pathway. *BMC Cancer*. 18, 1128. doi: 10.1186/s12885-018-5027-9

Wilkenfeld, S. R., Lin, C., and Frigo, D. E. (2018). Communication between genomic and non-genomic signaling events coordinate steroid hormone actions. *Steroids* 133, 2–7. doi: 10.1016/J.STEROIDS.2017.11.005

Xu, J., Wang, Y., Zhang, Y., Dang, S., and He, S. (2018). Astemizole promotes the anti-tumor effect of vitamin D through inhibiting miR-125a-5p-meidated regulation of VDR in HCC. *BioMed. Pharmacother.* 107, 1682–1691. doi: 10.1016/j.biopha.2018.08.153

Yang, H., Ma, L., Wang, Y., Zuo, W., Li, B., Yang, Y., et al. (2018). Activation of ClC-3 chloride channel by 17β-estradiol relies on the estrogen receptor α expression in breast cancer. *J. Cell. Physiol.* 233 (2), 1071–1081. doi: 10.1002/jcp.25963

Yee, N. S. (2015). Roles of TRPM8 ion channels in cancer: proliferation, survival, and invasion. *Cancers* 7 (4), 2134–2146. doi: 10.3390/cancers 7040882

Young, M. R. I., and Xiong, Y. (2018). Influence of vitamin D on cancer risk and treatment: why the variability? *Trends In Cancer Res.* 13, 43–53.

Zhang, C., Bosch, M. A., Qiu, J., Rønnekleiv, O. K., and Kelly, M. J. (2015). Rapid communication: 17 b-estradiol increases persistent Na^+ current and excitability of AVPV/PeN kiss1 neurons in female mice. *Mol. Endocrinol.* 29 (4), 518–527. doi: 10.1210/me.2014-1392

Zhuang, L., Peng, J. B., Tou, L., Takanaga, H., Adam, R. M., Hediger, M. A., et al. (2002). Calcium-selective ion channel, CaT1, is apically localized in gastrointestinal tract epithelia and is aberrantly expressed in human malignancies. *Lab. Invest.* 82 (12), 1755–1764. doi: 10.1097/01.LAB.0000043910.41414.E7

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 Restrepo-Angulo, Bañuelos and Camacho. 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 Pharmacology

ORIGINAL RESEARCH
published: 28 February 2020
doi: 10.3389/fphar.2020.00143

A Compartmentalized Reduction in Membrane-Proximal Calmodulin Reduces the Immune Surveillance Capabilities of CD8$^+$ T Cells in Head and Neck Cancer

*Ameet A. Chimote[1], Vaibhavkumar S. Gawali[1], Hannah S. Newton[1], Trisha M. Wise-Draper[2] and Laura Conforti[1]**

[1] Division of Nephrology, Department of Internal Medicine, University of Cincinnati, Cincinnati, OH, United States, [2] Division of Hematology Oncology, Department of Internal Medicine, University of Cincinnati, Cincinnati, OH, United States

OPEN ACCESS

Edited by:
Elena Adinolfi,
University of Ferrara, Italy

Reviewed by:
Hai Minh Nguyen,
University of California,
Davis, United States
Ildikò Szabò,
University of Padova, Italy

***Correspondence:**
Laura Conforti
laura.conforti@uc.edu

Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: 08 October 2019
Accepted: 04 February 2020
Published: 28 February 2020

Citation:
Chimote AA, Gawali VS, Newton HS, Wise-Draper TM and Conforti L (2020) A Compartmentalized Reduction in Membrane-Proximal Calmodulin Reduces the Immune Surveillance Capabilities of CD8$^+$ T Cells in Head and Neck Cancer. Front. Pharmacol. 11:143. doi: 10.3389/fphar.2020.00143

The limited ability of cytotoxic CD8$^+$ T cells to infiltrate solid tumors and function within the tumor microenvironment presents a major roadblock to effective immunotherapy. Ion channels and Ca^{2+}-dependent signaling events control the activity of T cells and are implicated in the failure of immune surveillance in cancer. Reduced KCa3.1 channel activity mediates the heightened inhibitory effect of adenosine on the chemotaxis of circulating T cells from head and neck squamous cell carcinoma (HNSCC) patients. Herein, we conducted experiments that elucidate the mechanisms of KCa3.1 dysfunction and impaired chemotaxis in HNSCC CD8$^+$ T cells. The Ca^{2+} sensor calmodulin (CaM) controls multiple cellular functions including KCa3.1 activation. Our data showed that CaM expression is lower in HNSCC than healthy donor (HD) T cells. This reduction was due to an intrinsic decrease in the genes encoding CaM combined to the failure of HNSCC T cells to upregulate CaM upon activation. Furthermore, the reduction in CaM was confined to the plasma membrane and resulted in decreased CaM-KCa3.1 association and KCa3.1 activity (which was rescued by the delivery of CaM). IFNγ production, also Ca^{2+}- and CaM-dependent, was instead not reduced in HNSCC T cells, which maintained intact cytoplasmic CaM and Ca^{2+} fluxing ability. Knockdown of CaM in HD T cells decreased KCa3.1 activity, but not IFNγ production, and reduced their chemotaxis in the presence of adenosine, thus recapitulating HNSCC T cell dysfunction. Activation of KCa3.1 with 1-EBIO restored the ability of CaM knockdown HD T cells to chemotax in the presence of adenosine. Additionally, 1-EBIO enhanced INFγ production. Our data showed a localized downregulation of membrane-proximal CaM that suppressed KCa3.1 activity in HNSCC circulating T cells and limited their ability to infiltrate adenosine-rich tumor-like microenvironments. Furthermore, they indicate that KCa3.1 activators could be used as positive CD8$^+$ T cell modulators in cancers.

Keywords: ion channels, T cells, head and neck cancer, KCa3.1, calmodulin, T cell function, T cell chemotaxis

INTRODUCTION

The immune system is the first line of defense against cancer. Indeed, in several solid malignancies, including head and neck squamous cell carcinomas (HNSCC), an increased infiltration of cytotoxic $CD8^+$ T cells is associated with a favorable patient prognosis and therapeutic response (Balermpas et al., 2014; Fang et al., 2017). However, the ability of $CD8^+$ T cells to infiltrate the tumor microenvironment (TME) and produce an anti-tumor response is often severely compromised (Uppaluri et al., 2008; Joyce and Fearon, 2015; Gurusamy et al., 2017). Ion channels have been implicated in this failure of immune surveillance in cancer (Chimote et al., 2017; Chimote et al., 2018).

Ion channels regulate multiple T lymphocyte functions including cytokine production, cytotoxicity, and motility, which are vital for generating an anti-tumor immune response (Cahalan and Chandy, 2009; Feske et al., 2012; Kuras et al., 2012; Zhong et al., 2013; Chimote et al., 2018). In particular, two K^+ channels, the voltage-dependent Kv1.3 and the Ca^{2+}-activated KCa3.1, regulate the electrochemical driving force for Ca^{2+} influx that is necessary for T cell effector functions (Cahalan and Chandy, 2009; Feske et al., 2012). Furthermore, KCa3.1 channels localize at the uropod of migrating human T cells and control chemotaxis (Kuras et al., 2012). Blockade of these channels suppresses T cell effector functions, motility, and chemotaxis (Cahalan and Chandy, 2009; Kuras et al., 2012). Indeed, Kv1.3 and KCa3.1 activity is reduced in tumor infiltrating lymphocytes (TIL) and circulating T cells of HNSCC patients, respectively, thus contributing to the failure of immune surveillance (Chimote et al., 2017; Chimote et al., 2018). Ultimately, restoring these channels' activity ameliorates T cell functionality, and overexpression of Kv1.3 decreases tumor burden and increases survival in tumor bearing mice (Eil et al., 2016; Chimote et al., 2018). Thus, understanding the causes of the abnormal behavior of these channels in cancer patients could lead to the discovery of new therapeutic targets for improved immune therapies in cancer.

Kv1.3 and KCa3.1 channels are suppressed by elements of the TME including hypoxia and adenosine (Chimote et al., 2012; Chimote et al., 2013; Chimote et al., 2017; Chimote et al., 2018). KCa3.1 channels, in particular, provide a link between adenosine and reduced T cell effector functions (Chimote et al., 2013). Adenosine, which is increased in TME of solid tumors, acts on T cells through the A_{2A} adenosine receptor ($A_{2A}R$), which in turn activates cAMP and PKA and, ultimately, inhibits KCa3.1, thus suppressing T cell motility and cytokine release (Chimote et al., 2013; Chimote et al., 2018). This immune suppressive pathway is particularly troublesome in HNSCC patients where $CD8^+$ T cells have an increased sensitivity to adenosine than healthy donors (HD) (Mandapathil et al., 2012; Chimote et al., 2018). The latter heightened response to adenosine is due to the diminished KCa3.1 function in HNSCC $CD8^+$ T cells, which is corrected by addition of KCa3.1 positive modulators (Chimote et al., 2018). Thus, defective KCa3.1 channel function can provide an explanation for the decreased immune surveillance in the adenosine-rich TME (Chimote et al., 2018). However, the mechanisms mediating these abnormalities in $CD8^+$ T cells of HNSCC patients are not known.

Calmodulin (CaM) gene expression has been shown to be altered in TIL of breast cancer patients (Gu-Trantien et al., 2013). CaM is a signaling molecule that controls multiple T cell functions including KCa3.1 activity which is triggered by an increase in cytosolic Ca^{2+} (Cahalan and Chandy, 2009; Sforna et al., 2018). CaM binds to the C-terminus of KCa3.1 and acts as a calcium sensor (Schumacher et al., 2001; Stocker, 2004; Adelman, 2016; Lee and Mackinnon, 2018; Sforna et al., 2018). CaM is constitutively bound to the channel in a Ca^{2+}-independent manner (Schumacher et al., 2001; Stocker, 2004; Adelman, 2016; Lee and Mackinnon, 2018). This pre-association allows for rapid channel gating when the intracellular Ca^{2+} concentration changes (Adelman, 2016). When intracellular Ca^{2+} increases, CaM undergoes conformational changes and causes the channel to open (Schumacher et al., 2001; Stocker, 2004; Lee and Mackinnon, 2018; Sforna et al., 2018). While it is known that CaM levels increase in activated healthy T cells, its protein abundance and functional implications in T cells of cancer patients, to our knowledge, have not yet been investigated (Colomer et al., 1993). In this study, we provide evidence of a compartmentalized reduction of CaM levels at the plasma membrane of $CD8^+$ T cells of HNSCC patients that selectively impacts KCa3.1 activity and chemotaxis. This mechanism may ultimately compromise immune surveillance in cancer.

MATERIALS AND METHODS

Human Subjects

Peripheral blood was obtained from 19 de-identified HNSCC patients in the age range of 47 to 84. The eligibility criteria for patient inclusion in the study were a positive diagnosis for HNSCC confirmed by tissue biopsy and no administration of radiation or chemotherapy before the time of drawing the blood (see **Table 1** for a summary of patient demographics and **Table S1** for clinical information). The data on the study subjects were collected and managed using Research Electronic Data Capture (REDCap) tools hosted at the University of Cincinnati. Peripheral blood was also drawn from seven age-matched (± 5 years) HD (five female and two male) in the age range of 41 to 67 years. Informed consent was obtained from all HNSCC patients and HDs. The study and informed consent forms were approved by the University of Cincinnati Institutional Review Board (IRB no. 2014-4755). As healthy controls, we also utilized discarded blood units from Hoxworth Blood Center (University of Cincinnati) for our studies. Age information about these samples are not available.

Reagents and Chemicals

Human serum, L-glutamine, adenosine, bovine brain calmodulin, 1-EBIO, sodium hydroxide, thapsigargin, poly-L-lysine, and Tween-20 were purchased from MilliporeSigma. Hepes, RPMI 1640, fetal bovine serum, penicillin, streptomycin, L-glutamine, Hepes, and phosphate-buffered saline (PBS) were obtained from Gibco (ThermoFisher). Collagen I, rat tail was obtained from Corning Inc. CXCL12 was obtained from R&D Systems. Stock solution of adenosine was prepared in sterile water. Stock solution of CXCL12 was prepared in sterile PBS containing 0.1% bovine serum albumin.

TABLE 1 | Demographics of HNSCC patients enrolled in the study.

Age (at the time of sample collection)	Years
Range	47–84
Mean	60
Variable	Number (%)
Gender	
Male	14 (74)
Female	5 (26)
Site	
Oral Cavity	6 (32)
Oropharynx	7 (37)
Larynx	5 (26)
Hypopharynx	1 (5)
Nasopharynx	0 (0)
Tumor stage	
T1	4 (21)
T2	8 (42)
T3	3 (16)
T4	4 (21)
Unknown	0 (0)
Nodal Status	
N0	6 (32)
N1	1 (5)
N2	10 (53)
N3	1 (5)
Unknown	1 (5)
ECOG (Eastern Cooperative Oncology Group) performance status	
0	8 (42)
1	4 (21)
2	2 (11)
Unknown	5 (26)
Smoking	
No (< 10 pk years)	9 (47)
Yes (> 10 pk years)	10 (53)
Alcohol	
No	16 (84)
Yes (> 5 drinks/week)	3 (16)
p16 status	
Positive	7 (37)
Negative	7 (37)
Unknown	5 (26)

HNSCC patients (n = 19) were enrolled in the study according to eligibility criteria. TNM classification was used to stage tumor size and nodal involvement. T1 to T4 refers to the size and invasion of the tumors. N1 to N3 refers to the number and location of the regional lymph nodes involved. Performance status was measured by ECOG score, a 0 to 5 scale that describes the daily living abilities of the patient. Smoking status was calculated by multiplying the number of packs of cigarettes smoked per day by the number of years the individual has smoked (pk years). A cut off of 10 pk years was used to differentiate smoking status.

Stock solutions of 1-EBIO and thapsigargin were prepared in dimethyl sulfoxide and used at 0.1% dilution.

Cell Isolation and *In Vitro* Activation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Paque density gradient centrifugation (GE Healthcare Bio-Sciences), as described previously (Chimote et al., 2018). $CD8^+$ T cells were isolated from PBMCs by negative selection using the EasySep Human $CD8^+$ T Cell Enrichment Kit (STEMCELL Technologies Inc.). The $CD8^+$ T cells were maintained in RPMI 1640 medium supplemented with 10% human serum, penicillin (200 U/ml), streptomycin (200 µg/ml), 1 mM L-glutamine, and 10 mM Hepes. For all experiments, cells were activated in a cell culture dish pre-coated with mouse anti-human CD3 antibody (10 µg/ml) (BioLegend) and mouse anti-human CD28 antibody (10 µg/ml) (BioLegend) for 72 to 96 h, except where specified.

Reverse Transcription Quantitative Polymerase Chain Reaction

Total RNA was isolated from resting and activated HD and HNSCC $CD8^+$ T cells using the E.Z.N.A. total RNA isolation Kit (Omega Biotek). 423 ng of RNA was used to synthesize complementary DNA (cDNA) using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (ThermoFisher). Predesigned primers for RT-qPCR were obtained using TaqMan Gene Expression Assays (Applied Biosystems, Thermo Fisher) to detect the expression of *CALM1* (assay ID: Hs00300085_s1), *CALM2* (assay ID: Hs00830212_s1), *CALM3* (assay ID: Hs00968732_g1), *18s rRNA* (assay ID: Hs99999901_s1), and *GAPDH* (assay ID: HS03929097_g1). The RT-qPCR was set up in a 96-well plate by adding 30 ng of cDNA, 1× TaqMan Gene Expression Master Mix (Applied Biosystems, ThermoFisher), and 1 µl of TaqMan Gene Expression Assay primers. All samples were run in technical replicates of quadruplicates or triplicates, as indicated in the individual figure or table legends. *18s rRNA* was used as an internal control. RT-qPCR was cycled in Applied Biosystems StepOne Real-Time PCR System (Applied Biosystems). C_T values were measured using StepOne software version 2.1 (Applied Biosystems). C_T values for *CALM1*, *CALM2*, and *CALM3* were normalized against measured C_T values for *18s rRNA*, and the $\Delta\Delta C_T$ values were calculated as described previously (Chimote et al., 2018). Relative quantity (RQ) values, representing the fold change in *CALM1*, *CALM2*, and *CALM3* gene expression in resting as compared to activated HD and HNSCC $CD8^+$ T cells, were calculated as the $2^{-\Delta\Delta C_T}$ values. To investigate the verity of *18s rRNA* as a housekeeping gene for our experiments, we measured *CALM1* expression in resting and activated $CD8^+$ T cells from a single HNSCC patient using either *18s rRNA* or *GAPDH* as housekeeping genes (**Table S2**). The C_T values for *CALM1* were normalized against measured C_T values for *18s rRNA*, as well against C_T values for *GAPDH* in resting and activated HNSCC $CD8^+$ T cells. We also normalized the C_T values for *GAPDH* against the C_T values of *18s rRNA*. RQ values were calculated as $2^{-\Delta\Delta C_T}$ values as described above and the results are presented in **Table S2**.

Immunofluorescence and Confocal Microscopy

2 X 10^5 activated $CD8^+$ T cells from HDs and HNSCC patients were plated on poly-L-lysine-coated coverslips in a four-well tissue culture plate, fixed in 1% paraformaldehyde, and blocked with a solution of PBS containing 10% FBS for 1 h at room temperature. Cells were then stained overnight at 4°C with either Alexa 488-conjugated mouse anti-human CD8 antibody (Clone SPM548, Novus Biologicals) or biotin-conjugated mouse anti-human KCa3.1 antibody (Clone 6C1, Alomone labs) diluted in blocking solution. The background staining of the Biotin conjugated KCa3.1 antibody is shown in **Figure S1**. The following day, coverslips labeled with the biotin-conjugated

primary were incubated with Alexa Fluor 488 conjugated-streptavidin secondary antibody (Biolegend) for 1 h at room temperature. All coverslips were then permeabilized with PBS + 0.2% Triton X-100 for 20 min at room temperature, blocked with blocking solution for 1 h, and stained with Alexa Fluor 647–conjugated mouse anti-human CaM antibody (clone 2D1, Novus Biologicals) diluted in blocking solution for 4 h at room temperature. Cells were washed thoroughly with PBS-T washing solution and coverslips were mounted on glass slides using Fluoromount G (eBiosciences, ThermoFisher). Cells were visualized by confocal microscopy (Zeiss LSM 710 laser scanning microscope, Zeiss GmbH) using a 63× water immersion objective lens at room temperature and the pinhole was set at 1 airy unit. Z-stack images of 0.75 µm thickness were acquired with the Zeiss Zen Image browser (Zeiss GmbH) using the "Multi Track" option of the microscope to exclude the cross-talk between the channels. Five to ten fields were imaged for each HD and HNSCC patient.

Image Analysis

Images were analyzed using ImageJ software (NIH, Bethesda, MD). Regions of interest (ROI) analysis: Briefly, in each field, the z-stack in the focal plane of the cell membrane (indicated by the presence of membrane localization of CD8 fluorescence) was visualized, and a ROI was drawn around the cell membrane and another ROI was drawn in the cytoplasmic region of individual T cells. The image threshold and background were adjusted and mean fluorescence intensities (mean gray values) for the CaM channels were measured for the membrane and cytoplasmic ROIs. The ratios of CaM fluorescence in the membrane and cytoplasmic ROIs were calculated for individual cells. For the experiments where cells were stained for KCa3.1, fluorescence intensity of KCa3.1 in HNSCC and HD T cells was measured in ROIs drawn around the cell membranes of individual cells.

Membrane and intracellular fluorescence measurement analyses of KCa3.1 and CaM were performed by comparing mean gray values (fluorescence intensity)-distance (µm) profiles (line scans) on acquired images of HNSCC and HD cells. The z-stacks in the focal plane of the cell membrane were identified on basis of distinct membrane KCa3.1 fluorescence. Two to three lines were drawn across each cell, and linescan analysis was performed to identify KCa3.1 and CaM expression using the plot profile function on ImageJ. The abscissa corresponding to outermost KCa3.1 fluorescence peaks on the linescan plot defined the cell surface, where the channel was present. Fluorescence intensities (gray values) for KCa3.1 and CaM at the cell membrane (the points of KCa3.1 fluorescence peaks) were measured in every linescan analysis performed.

Proximity Ligation Assay (PLA)

PLA experiments were performed using Duolink *In Situ* Detection Reagents Orange (DUO92102, Millipore Sigma) as described by us previously (Hajdu et al., 2015). Activated $CD8^+$ T cells from HDs and HNSCC patients were seeded on poly-L-lysine-coated coverslips, fixed with 4% paraformaldehyde, permeabilized with PBS + 0.2% Triton X-100, blocked with PLA blocking solution (Millipore Sigma) for 1 h, and stained with mouse anti-human KCa3.1 (Clone 6C1, Alomone labs) and rabbit anti-human CaM antibodies (clone SJ16-09, Novus Biologicals) diluted in antibody dilution buffer (Millipore Sigma) for 3 h at room temperature. The specificity of the monoclonal rabbit anti-human CaM antibody is shown in **Figure S2**. PLA procedure was done according to the manufacturer's protocol. After the final washes, the cells were labeled with Alexa Fluor 647-conjugated rabbit anti-human CD8 alpha antibody (clone EP1150, Abcam) diluted in antibody dilution buffer for 2 h at room temperature. The coverslips were washed, stained with 4′, 6-diamidino-2-phenylindole (DAPI, 1 µg/ml, ThermoFisher) to label the cell nuclei and mounted on glass slides as described earlier. Confocal images were obtained on a Zeiss LSM 710 microscope with 100× oil immersion lens at room temperature and the pinhole was set at 1 airy unit. Z-stack images of 0.75 µm thickness were acquired with the Zeiss Zen Image browser (Zeiss GmbH) using the "Multi Track" option of the microscope to exclude the cross-talk between the channels. Five to ten fields were imaged for each HD and HNSCC patient. Successful interaction by PLA between the KCa3.1 and CaM pairs were detected as bright fluorescent spots. As technical controls for non-specific binding, PLA was performed by omitting either the KCa3.1 or CaM antibodies or PLA probes (**Figure S3**).

Quantitation of PLA signals in the acquired images was performed using ImageJ. Briefly, the image threshold and background were adjusted for each image and a ROI was drawn around the cell membranes (identified by CD8 staining) and the positive PLA signals (dots) within the ROI were quantified by particle analysis, normalized to the number of cells counted in each field, and presented as the number of dots counted per cell membrane. For three-dimensional visualization of the PLA signal on the membrane, the z-stacks from representative PLA confocal images of T cells from HD and HNSCC individuals acquired as described earlier were reconstructed into 3D using Imaris image analysis software (Bitplane AG, Zurich, Switzerland).

Electrophysiology

KCa3.1 and Kv1.3 currents were recorded from activated $CD8^+$ T cells in whole-cell voltage clamp configuration using an AxoPatch 200B Amplifier (Molecular devices). Pipettes were formed from Borosilicate glass (TW150F-4, World Precision Instruments) with a P-97 horizontal puller (Sutter Instruments) and had a resistance between 4 and 5 MΩ when filled with intracellular pipette solution consisted of (mM) 145 K-Aspartate, 2 $MgCl_2$, 8.5 $CaCl_2$, 10 EGTA, and 10 HEPES. The pH value was set to 7.2 using TRIS buffer. Unless stated otherwise 1 µM free Ca^{2+} concentration was maintained inside the pipette solution. The external solution consisted of (mM) 160 NaCl, 4.5 KCl, 1 $MgCl_2$, 10 HEPES; the pH value was set to 7.4 using NaOH. In a subset of experiments we utilized pipette solution with 3 and 10 µM free Ca^{2+} concentration. Free Calcium

concentration was calculated using a program developed by Stanford University. (http://web.stanford.edu/~cpatton/webmaxcS.htm).

Data were acquired using pCLAMP 8.0 software (Molecular Devices) through a 16-bit A-D/D-A interface (Digidata1320A, Molecular Devices). Data were low pass filtered at 2 kHz and digitalized at 100 kHz. The electrophysiological protocol to record KCa3.1 currents implemented a ramp pulse from −120 to +50 mV from a holding potential of −70 mV every 15 s. The macroscopic conductance (G) of KCa3.1 channels was calculated as a ratio of the liner fraction of macroscopic current slope to the slope of ramp voltage stimulus after subtraction of the leak current (Chimote et al., 2018). The KCa3.1 slope conductance was calculated using linear regression in the voltage range between −100 and −80 mV to avoid contamination by the Kv1.3 current. Kv1.3 currents were determined from the same ramp protocol at +50 mV after subtraction of the KCa3.1 current extrapolated by linear regression. CaM was dissolved in pipette solution to make a final concentration of 50 μM. While performing electrophysiology experiments on HD T cells transfected with either si*CALM1* or scr-RNA, the cells expressing GFP fluorescence were evaluated.

Intracellular Ca^{2+} Measurements

$CD8^+$ T cells from HD or HNSCC patients were plated on poly-L-lysine coated coverslips, loaded with 1 μM of Fura-2/AM (ThermoFisher) and Fura-2 intensities were measured using InCytIm2 Ca^{2+} imaging system (Intracellular Imaging, Cincinnati, OH, USA) as previously described (Robbins et al., 2005; Kuras et al., 2012). Briefly, the cells were first perfused with a 0 mM Ca^{2+} solution for 5 min, then they were perfused for additional 5 min with the 0 mM Ca^{2+} solution containing 1 μM thapsigargin (MilliporeSigma) and lastly, cells were perfused with 0.5 mM Ca^{2+} solution for 10–15 min. The 0 mM Ca^{2+} solution contained (in mM): 155 NaCl, 4.5 KCl, 1 $MgCl_2$, 10 HEPES, 10 glucose, 2 EGTA, pH 7.4; while the 0.5 mM Ca^{2+} solution contained (in mM): 155 NaCl, 4.5 KCl, 2.5 $MgCl_2$, 10 HEPES, 10 glucose, 0.5 $CaCl_2$, pH 7.4. A standard curve was used to correlate the measured ratiometric Fura-2 intensities (340/380 nm ratio) to the intracellular Ca^{2+} levels, as per the manufacturer's instructions. Change in Ca^{2+} (ΔCa^{2+} flux) was calculated as the difference in the measured Fura-2 ratio from its peak value in the presence of 0.5 mM Ca^{2+} to the baseline measured after addition of thapsigargin.

siRNA

Silencer® Pre-designed siRNA against *CALM1* gene (siRNA ID 146695, catalog number AM51331) and scrambled sequence siRNA (Silencer® Negative Control #1 siRNA, catalog number AM4611) were obtained from ThermoFisher.

Transfection

$1–6 \times 10^6$ $CD8^+$ T cells that were isolated from HD and activated for 72 h were transfected using the Amaxa™ P3 Primary Cell 4D-Nucleofector™ X Kit (Lonza Cologne GmbH). 100 nM si*CALM1* or scr- RNA were cotransfected with 2 μg GFP (pMAX GFP supplied in the Amaxa Nucleofector Kit) using program E0-115 (program: "T cell stimulated") using a 4D-Nucleofector™ unit (Lonza Cologne GmbH) as per manufacturer's instructions. The transfected cells were incubated overnight at 37° C and further activated for 24 h with anti-CD3 and anti-CD28 antibodies (total 96 h activation). The efficiency of transfection was ~30% as determined by cells expressing GFP fluorescence. The cells that expressed GFP were considered to be transfected successfully and were used for electrophysiology, flow cytometry, and chemotaxis experiments.

Flow Cytometry

$CD8^+$ T cells ($\sim 1 \times 10^6$ cells per condition) were stained with Zombie Aqua Fixable Viability Kit (Biolegend) according to manufacturer's instructions, fixed with 4% paraformaldehyde (Affymetrix, Thermo Fisher Scientific), stained with ATTO 488–conjugated mouse anti-human KCa3.1 extracellular antibody (clone 6C1, Alomone Labs) without permeabilization. For staining with CaM, fixed cells were permeabilized with 0.1% Triton X-100 (MilliporeSigma) and stained with Alexa Fluor 647–conjugated mouse anti-human CaM antibody (clone 2D1, Novus Biologicals). To test for T cell activation, $CD8^+$ T cells were stained live with APC-Fire™750–conjugated mouse anti-human anti-CD69 antibody (Clone FN50, Biolegend) and then fixed with 1% paraformaldehyde. For intracellular IFNγ staining, PBMC were isolated from HD and were stimulated for 24 h with cell activation cocktail (Biolegend) and were transfected with either si*CALM1* or scr-RNA along with GFP as described earlier. Post-transfection, cells were stimulated for 3 h with cell activation cocktail in the presence of brefeldin A (BD Biosciences) in the presence or absence of 1-EBIO. We also isolated PBMC from HD's and HNSCC individuals and stimulated them for 3 h in the presence of brefeldin A in the presence or absence of 1-EBIO. Unstimulated HD and HNSCC PBMC were also incubated in the presence of brefeldin A. All the cells were first stained with Zombie Aqua Fixable Viability Kit according to the manufacturer's instructions, surface stained with Pacific Blue conjugated mouse-anti human anti-CD3 antibody (Clone OKT3, Biolegend), fixed and permeabilized using BD Cytofix/Cytoperm kit (BD Biosciences). The permeabilized cells were then stained with Brilliant violet 605 conjugated mouse anti-human anti-IFNγ antibody (Clone 4S.B3, BioLegend). For all flow cytometry experiments, data were collected on an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo software (FlowJo LLC). In experiments performed on T cells transfected with si*CALM1* or scr-RNA, cells that were gated on the GFP positive population (indicative of successful transfection) were used for data evaluation.

Chemotaxis

Three-dimensional chemotaxis was performed using the μ-Slide Chemotaxis assay (ibidi GmbH) as described previously (Chimote et al., 2018). Briefly, $\sim 1 \times 10^6$ $CD8^+$ T cells were activated for 72 h and then transfected with either si*CALM1* or scr-RNA along with GFP and further activated for 24 h. The cells were then incorporated in a type I rat tail collagen gel (Corning) as per the manufacturer's instructions and added to the central observation chamber of the μ-Slide Chemotaxis. CXCL12 was used as the chemokine for all chemotaxis experiments and 8 μg/ml CXCL12 was added to the migration medium in the reservoir to the left of the observation chamber to generate a chemokine gradient to measure the baseline chemotaxis as described previously (Chimote et al., 2018). To assess the effects of adenosine on chemotaxis, we established simultaneous

adenosine and chemokine gradients in a separate chamber as reported previously (Chimote et al., 2018). The slide was then mounted on the stage of an inverted Zeiss LSM 710 microscope (Carl Zeiss Microscopy GmbH) equipped with a 37°C incubator. Time-lapse video microscopy was performed to record cell migration and images were acquired every 3 s for up to 3 h in the bright field and GFP channels. Cells that were successfully transfected were identified by the presence of a strong intracellular GFP signal and cell tracking was performed using the "Manual Tracking plugin" on ImageJ software (National Institutes of Health), and the data were analyzed using the Chemotaxis and Migration Tool (ibidi GmbH). Ten to 15 transfected (GFP positive) cells were tracked per condition. Chemotactic effect was assessed by measuring the center of mass (COM, the average position along the relevant axis that the cells reached by the end of the experiment). A positive chemotaxis was defined if the cells migrated along the CXCL12 gradient (y axis) that is, a Y-COM > 0 indicated a positive chemotaxis (Chimote et al., 2018).

Statistical Analysis

Statistical analyses were performed using Student's t test (paired or unpaired), Mann-Whitney rank sum test (in experiments where samples failed normality), and ANOVA as indicated. *Post hoc* testing on ANOVA was done by multiple pairwise comparison procedures using the Holm-Sidak method. Statistical analysis was performed using SigmaPlot 13.0 (Systat Software Inc.). P value of less than or equal to 0.05 was defined as statistically significant.

RESULTS

Expression of Calmodulin Is Reduced in HNSCC CD8$^+$ T Cells

Experiments were performed to assess whether CaM expression is altered in CD8$^+$ T cells of HNSCC patients. CaM is a small protein of 148 amino acids (~17kD), which is encoded by three genes *CALM1*, *CALM2*, and *CALM3*, and downregulation of either of these genes can result in decreased CaM protein levels (Colomer et al., 1993; Marshall et al., 2015). We investigated CaM mRNA and protein levels in resting and activated T cells in healthy donors (HD) and HNSCC patients (**Figure 1**). All experiments were conducted on CD8$^+$ T cells that were isolated from HD and HNSCC patients and activated *in vitro* using anti-CD3 and anti-CD28 antibodies for 72 to 96 h, unless otherwise specified. We have previously reported that there is no difference in the activation status of HNSCC and HD T cells (Chimote et al., 2018). KCa3.1 and CaM expression is increased in activated healthy T cells (Colomer et al., 1993; Ghanshani et al., 2000; Cahalan and Chandy, 2009). T cells from HD showed an increase in *CALM1*, *CALM2*, and *CALM3* expression on activation (**Figure 1A**), whereas HNSCC T cells failed to upregulate *CALM1*, *CALM2*, and *CALM3* mRNAs (**Figure 1B**). Essentially, *CALM1* and *CALM2* mRNA levels were significantly reduced in resting HNSCC T cells as compared to resting HD T cells, whereas *CALM1*, *CALM2*, and *CALM3* mRNA levels were significantly lower in activated HNSCC T cells as compared to their activated healthy counterparts (**Figures 1C, D**). In the qPCR experiments, we used *18s rRNA* as the housekeeping gene. Comparable results were obtained with *GAPDH* as the housekeeping gene (**Table S2**). Measurement of CaM protein levels by flow cytometry confirmed that HD T cells increased CaM post-activation, while CaM decreased after activation in HNSCC T cells (**Figures 1E, F**). CaM protein abundance was not significantly different in resting HD and HNCC T cells (**Figure 1G**), while in activated HNSCC T cells it was 31% lower than their healthy counterparts (**Figure 1H**).

HNSCC T Cells Display a Compartmentalized Decrease in Membrane CaM

A reduction in CaM could have enormous implications in T cell function as CaM oversees the activity of many molecules (Stocker, 2004; Berchtold and Villalobo, 2014; Lee and Mackinnon, 2018). Thus, we conducted experiments to determine whether the reduction of CaM in HNSCC T cells spanned all cell compartments. Confocal microscopy experiments were conducted in CD8$^+$ T cells from HNSCC patients and from HD to determine the CaM membrane and cytoplasmic abundance. Cells were fixed, stained with anti-CD8 antibody to label the membrane (prior to permeabilization), and then, stained post-permeabilization with anti-CaM antibody (**Figure 2A**). We evaluated CaM levels in HD and HNSCC T cells by measuring the fluorescence intensity of CaM at the cell membrane (defined by the CD8 staining) as well as in the cytoplasm. The distribution of membrane CaM differs in HNSCC vs HD T cells (**Figures 2B, D**). Membrane CaM was lower in HNSCC T cells as compared to their healthy counterparts by ca. 41% (**Figure 2B**), while the cytoplasmic CaM was 13% higher in HNSCC T cells as compared to their healthy counterparts (**Figure 2C**). CaM levels in the membranes of HD T cells were 51% higher than in the cytoplasm (with a membrane: cytoplasm CaM fluorescence ratio of 1.5); in HNSCC T cells they were 10% lower (membrane: cytoplasm CaM fluorescence ratio of 0.9) (**Figure 2D**). On the other hand, activated CD8$^+$ T cells from HD and HNSCC individuals stained with extracellular anti-KCa3.1 (with no permeabilization, **Figure 2E**) showed similar membrane abundance of KCa3.1, as reported previously (Chimote et al., 2018) (**Figure 2F**). Overall, these data indicate that HNSCC T cells express less CaM than HD T cells, and this difference is confined to the membrane compartment. This, in turn, suggests that in HNSCC T cells, fewer KCa3.1 channels may be coupled to CaM than in HD T cells. It also suggests that the defect in CaM of HNSCC T cells may not impact other functions that rely on cytoplasmic CaM.

HNSCC T Cells Show Lower Association of Membrane KCa3.1 with CaM and Decreased KCa3.1 Activity

We performed confocal microscopy experiments to measure the association of CaM with KCa3.1 at the plasma membrane. CD8$^+$ T cells from HD and HNSCC individuals were stained with extracellular anti-KCa3.1 (with no permeabilization) and

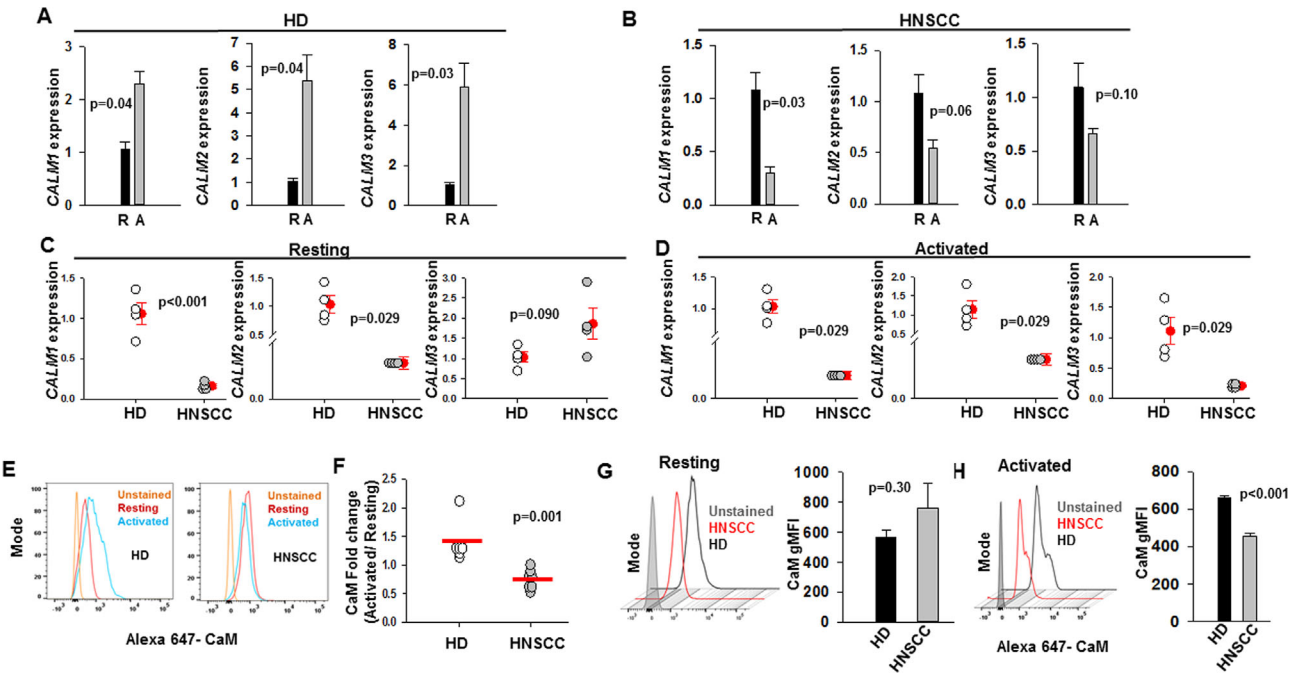


FIGURE 1 | Decreased calmodulin expression in HNSCC T cells. **(A, B)** *CALM1*, *CALM2*, and *CALM3* expression in resting, "R" and activated, "A" T cells in HD (panel **A**) and HNSCC (panel **B**) patients were quantified by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Data are the fold change in *CALM1*, *CALM2*, and *CALM3* expression relative to *18S rRNA* expression. The data were normalized to mean resting *CALM1*, *CALM2*, and *CALM3* gene expression in HD and HNSCC. For panels **(A, B)** bars represent mean ± SEM from four HD and four HNSCC patients, with samples run in quadruplicate. **(C, D)** *CALM1*, *CALM2*, and *CALM3* expression in resting **(C)** and activated **(D)** T cells in HD and HNSCC patients were quantified by RT-qPCR. Data are the fold change in *CALM1*, *CALM2*, and *CALM3* expression relative to *18S rRNA* expression. The data were normalized to mean *CALM1*, *CALM2*, and *CALM3* expression in HD T cells for the resting and activated conditions. For panels **(C, D)** the solid red circles and error bars represent mean ± SEM of four HD and four HNSCC patients, with samples run in quadruplicate. **(E)** Flow cytometry histograms showing CaM expression in resting and activated T cells from representative HD and HNSCC patients. **(F)** Fold change in CaM geometric mean fluorescence intensity (gMFI) in T cells from HD (n = 6) and HNSCC patients (n = 7) calculated as a ratio of CaM gMFI in activated cells over CaM gMFI in resting cells. Horizontal red line represents the mean for each group. **(G)** Representative flow cytometry overlay histogram showing CaM expression in resting T cells from one HD and one HNSCC patient. The bar graph on the right shows gMFI for CaM expression in resting T cells from HD (n = 6) and HNSCC patients (n = 7). Bars represent mean ± SEM. **(H)** Representative flow cytometry overlay histogram showing CaM expression in activated T cells from one HD and one HNSCC patient. CaM expression was measured in live cell population identified by exclusion from Zombie Aqua live/dead stain. The bar graph on the right shows gMFI for CaM expression in activated T cells from HD (n = 3) and HNSCC patients (n = 3). Data in **(A, B)** were analyzed by paired student's t-test; data in **(C)** for *CALM1* and *CALM3* expression were analyzed by student's t-test, whereas data for *CALM2* expression were analyzed by Mann-Whitney rank sum test, data in **(D, F)** were analyzed by Mann Whitney rank sum test; data in **(G, H)** were analyzed by Student's t-test.

intracellular anti-CaM (after permeabilization) antibodies (**Figure S4**). The membrane distribution of KCa3.1 and CaM was determined by linescan analysis. We observed that in the membranes of HNSCC cells, the fluorescence intensity of CaM (**Figure 3A**, magenta) corresponding to the KCa3.1 peaks (green), was lower than that in HD T cells. In some HNSCC T cells, we observed KCa3.1 proteins with no, or undetectable, associated CaM. Indeed, while KCa3.1 peak fluorescence intensities at the membrane were comparable in HD and HNSCC T cells, there was a 37% reduction in membrane KCa3.1-associated CaM (CaM fluorescence intensity corresponding to the KCa3.1 fluorescence intensity peaks, see *Materials and Methods*) in HNSCC T cells (**Figures 3B, C**). We then performed proximity ligation assay (PLA) to confirm whether the physical membrane association between CaM and KCa3.1 channels was reduced in HNSCC T cells. This assay detects the interaction between proteins that reside ≤ 40-nm through species-specific antibodies, each labeled with unique short oligonucleotides, which amplify when the two antibodies are in close proximity. The interaction is detected with a fluorescently labeled oligonucleotide that hybridizes to the amplicons, which can be subsequently visualized and quantified as discrete dots (PLA signals) by microscopy (Hajdu et al., 2015). As shown in **Figure 3D**, both HNSCC and HD T cells showed positive PLA signals (dots) at the cell membrane. However, analysis of the confocal images revealed that the number of dots/cell membrane in HNSCC T cells was reduced by 57% as compared to HD T cells (**Figures 3E, F**). These data confirm that KCa3.1 channels in the membranes of HNSCC T cells have a lower association with CaM. We performed further experiments to assess whether the decreased KCa3.1 function that we observed in HNSCC $CD8^+$ T cells is due to decreased availability of CaM (Chimote et al., 2018). Patch-clamp experiments confirmed that KCa3.1 activity

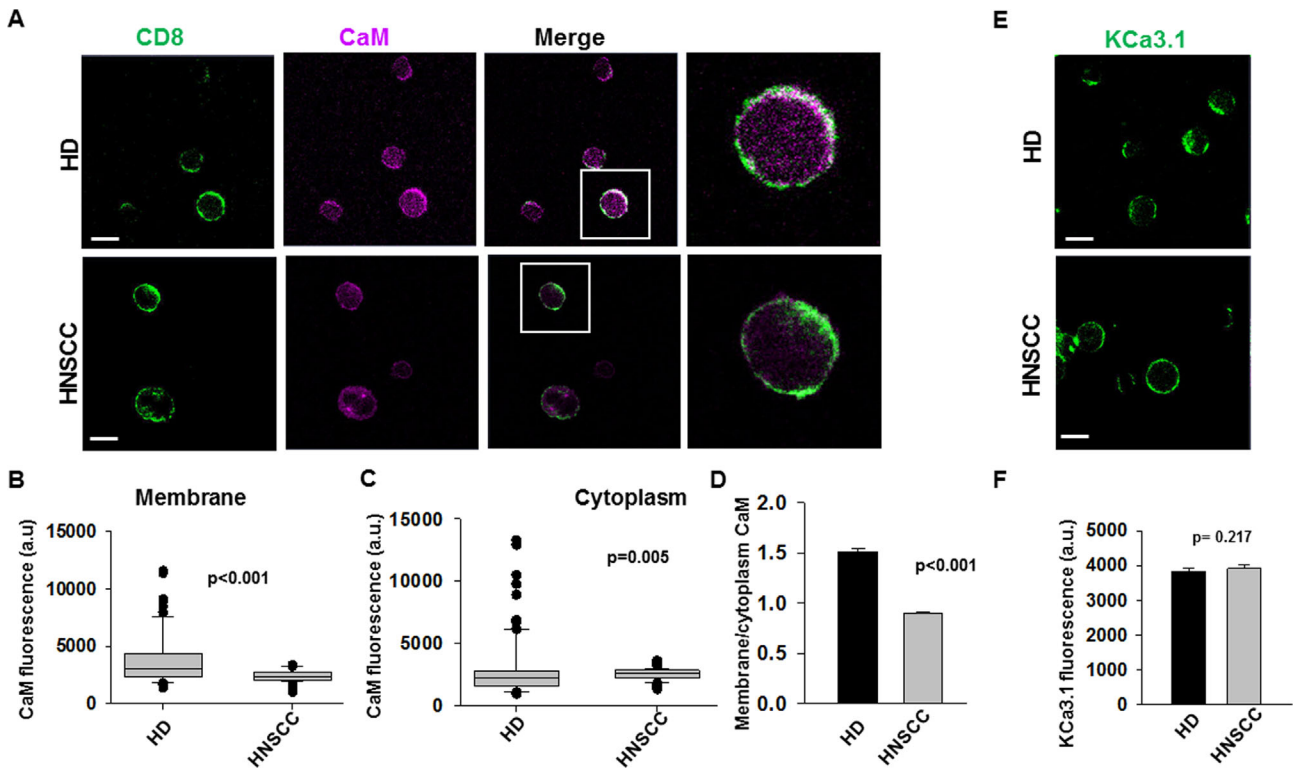


FIGURE 2 | Decreased membrane CaM in HNSCC T cells. **(A)** Representative confocal images of HD and HNSCC T cells stained for CD8 (green; no permeabilization) and CaM (magenta, after permeabilization). Scale bars indicate 10 μm. Representative individual cells highlighted (box) in the merged images are magnified in the extreme right panels. Co-localization is indicated by white color. **(B, C)** Fluorescence intensities of CaM in the membrane **(B)** and cytoplasm **(C)** of HD and HNSCC T cells were measured as described in *Materials and Methods*. Data represented by a box and whisker plot. The horizontal line represents median value of CaM fluorescence intensities for each group, while the first and third quartiles are represented in the top and bottom boxes respectively. **(D)** Ratio of CaM fluorescence at the cell membrane and cytoplasm of HD and HNSCC T cells. Bars represent mean ± SEM **(E)** Representative confocal images of HD and HNSCC T cells stained for KCa3.1 (green). Scale bars indicate 10 μm. **(F)** Fluorescence intensities of KCa3.1 in the membranes of HD and HNSCC T cells. Bars represent mean ± SEM of 117 cells from 3 HD and 61 cells from 3 HNSCC patients. Shown in **(B–D)** are values for 96 cells from 3 HD and 110 cells from 3 HNSCC patients. Data in **(B–D, F)** were analyzed by Mann Whitney rank sum test.

(defined by the whole-cell KCa3.1 conductance, G) was significantly lower in activated HNSCC T cells as compared to their healthy counterparts as previously shown (**Figures 4A, B**) (Chimote et al., 2018). However, intracellular addition of CaM, *via* the patch pipette, increased the KCa3.1 activity of HNSCC T cells to the level of HD T cells (**Figures 4A, B**). Conversely, we observed that addition of CaM did not increase KCa3.1 activity in HD T cells (**Figures 4A, B**). Addition of CaM had no effect on Kv1.3 activity (defined as peak current) in either HNSCC or HD T cells (**Figure 4C**). There was no change in cell capacitance (a measure of cell size) in the controls or CaM-treated cells either in HNSCC or HD T cells (**Table S3**). All patch-clamp experiments were performed with an internal pipette solution containing 1 μM free-Ca^{2+}. To assess whether a decrease in Ca^{2+} sensitivity of the channels also contributed to the decrease in KCa3.1 function in HNSCC T cells, we measured KCa3.1 conductance in HNSCC T cells in the presence of incremental intracellular free Ca^{2+} concentrations (1 μM, 3 μM, and 10 μM). There was no effect of intracellular Ca^{2+} on the KCa3.1 conductance in HNSCC T cells (**Figure 4D**). Thus, these findings indicate that the decreased KCa3.1 activity in HNSCC T cells, which limits tumor infiltration, is due to an intrinsic defect in CaM (Chimote et al., 2018). We then investigated whether this defect in CaM observed in HNSCC T cells inhibits other Ca^{2+} and CaM-dependent functions like IFNγ production. We found no decrease in INFγ production in activated HNSCC T cells (**Figure 5A**). We also found no alterations in their Ca^{2+} fluxing abilities (**Figure 5B**) which, combined with the intact cytoplasmic CaM (**Figure 2C**), support an efficient IFNγ production. Overall, these data indicate that the alteration in CaM of HNSCC T cells selectively impacted T cell functions that depend on KCa3.1 activity.

Knockdown of CALM1 in HD T Cells Reduces KCa3.1 Activity and Increases Their Sensitivity to Adenosine

We then conducted experiments to study whether lowering CaM expression in HD T cells would result in a phenotype comparable to that of HNSCC T cells. We knocked down the

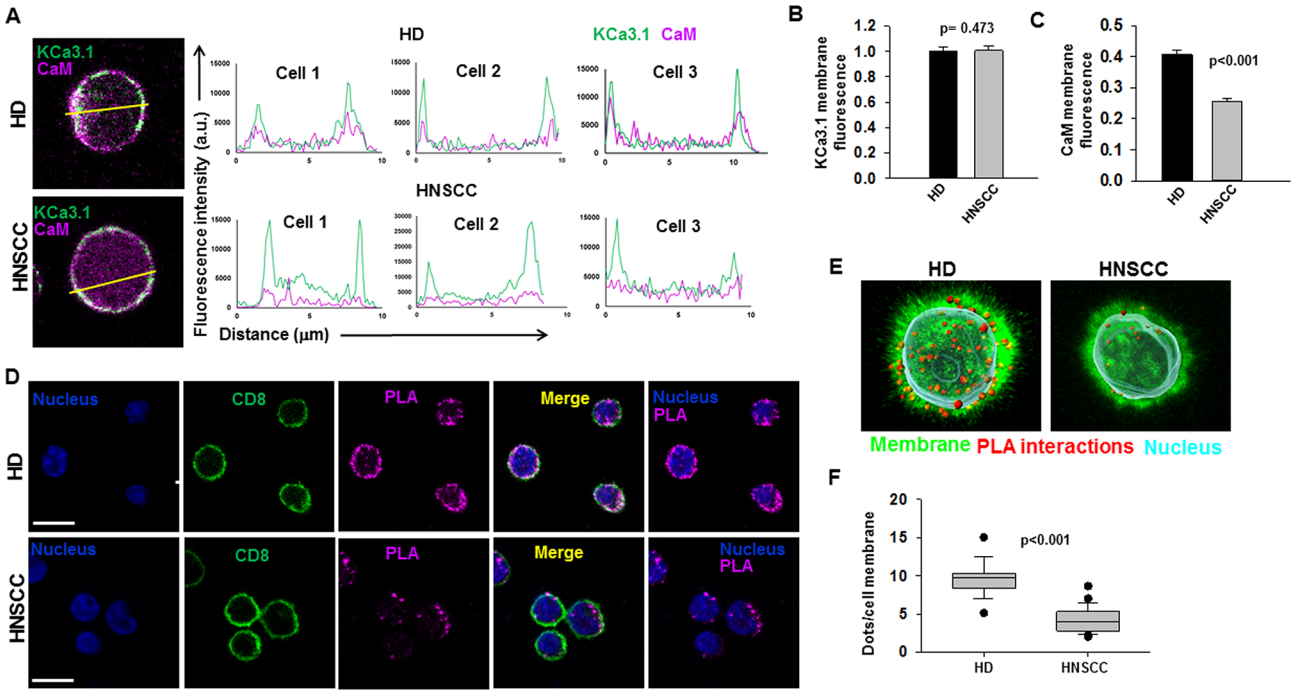


FIGURE 3 | Reduced association of KCa3.1 with CaM in HNSCC T cells **(A)** Linescan analysis of KCa3.1 and CaM association at the cell membrane. (Left) Magnified representative confocal images of HD and HNSCC T cells stained for KCa3.1 (green) and CaM (magenta) from the merged images in **Figure S4** (box). Lines were drawn across the cells for linescan analysis, and representative lines drawn for a linescan are shown. (Right) Line scan analyses were generated using ImageJ software and are plotted as KCa3.1 (green line) and CaM (magenta line) fluorescence intensity (y-axis) *vs.* the length of the lines drawn across the merged cells (distance from the starting point of the line, μm, x-axis). Shown here are linescan graphs from three representative HD and HNSCC T cells, wherein the outmost KCa3.1 (green) peaks correspond to the cell membrane. Correspondence of the peaks for green (KCa3.1) and magenta (CaM) lines is indicative of their colocalization. **(B)** Comparison of KCa3.1 membrane expression in HD and HNSCC T cells. KCa.31 fluorescence intensities were measured at the cell surface (points corresponding to KCa3.1 fluorescence peaks on the cell membrane) in linescan plots **(A)**. The data were normalized to mean KCa3.1 fluorescence intensity measured in HD T cells. **(C)** Comparison of CaM membrane-associated expression in HD and HNSCC T cells. CaM fluorescence intensities were measured at the cell surface (points corresponding to KCa3.1 fluorescence peaks on the cell membrane) in linescan plots **(A)**. The data were normalized to mean KCa3.1 fluorescence intensity measured in HD T cells, which represents the proportion of CaM associated with membrane KCa3.1. For **(B, C)**, bars represent mean ± SEM for data from 108 points (corresponding to KCa3.1 fluorescence peaks in the linescans) analyzed in 18 cells from 3 HD, and 120 points analyzed in 20 cells from 3 HNSCC patients. **(D)** Interaction between KCa3.1 and CaM in cell membranes of HD and HNSCC T cells. Confocal images of representative cells from PLA experiments performed in HD (top) and HNSCC (bottom). Cells were incubated with rabbit anti-human CaM and mouse anti-human KCa3.1 antibodies, after which PLA probe–ligated secondary antibodies were then added and PLA was performed as described in *Materials and Methods*. Positive PLA signals showing single protein interactions between KCa3.1 and CaM are visualized as magenta fluorescent dots. Cell membranes are labeled with CD8 antibodies (green) and nuclei are labeled with DAPI (blue). Scale bar, 10 μm. **(E)** 3D views of the PLA dots (red) in relation to the nucleus (DAPI, cyan) and membrane (CD8, green) in a representative HD (left) and HNSCC (right) T cell. **(F)** Average PLA signals. PLA signals in the cell membranes of in HD and HNSCC T cells were quantified as number of dots per cell membrane and are represented as a box plot. The dots per cell membrane are reported as median (horizontal line), first (top box), and third quartiles (bottom box). Shown are values for 185 cells from 3 HD and 214 cells from 4 HNSCC patients. For **(B, C)**, significance was determined by Mann Whitney rank sum test, while the significance for **(F)** was determined by Student's t-test.

CALM1 gene in activated $CD8^+$ T cells from HD using a siRNA against *CALM1* (si*CALM1*) while scrambled sequence siRNA (scr-RNA) was used as control. Transfection with si*CALM1* decreased CaM expression in activated HD T cells by 30% as compared to scr-RNA-transfected cells (**Figure 6A**); a reduction in CaM expression comparable to that observed in activated HNSCC T cells (**Figure 1H**). Patch-clamp experiments showed that si*CALM1* reduced KCa3.1 activity by 30% (**Figure 6B**), while Kv1.3 activity and cell capacitance remained unchanged (**Figure 6C**, **Table S3**). Silencing *CALM1* did not significantly decrease KCa3.1 and CD69 (T cell activation marker) protein levels (**Figure S5**). Thus, silencing *CALM1* reproduced in HD T cells the K^+ channel phenotype previously reported for HNSCC T cells: low KCa3.1 activity, and normal Kv1.3 activity (Chimote et al., 2018). We conducted further experiments to see whether the knockdown of *CALM1* in HD T cells and the consequent decrease in their KCa3.1 function compromised other T cell functions. Low KCa3.1 activity has been implicated in the increased sensitivity of HNSCC T cells to adenosine that led to a heightened inhibitory effect of adenosine on chemotaxis (Chimote et al., 2018). Hence, we conducted 3D chemotaxis experiments to measure the effect of adenosine in si*CALM1*-transfected HD T cells (scr-RNA-

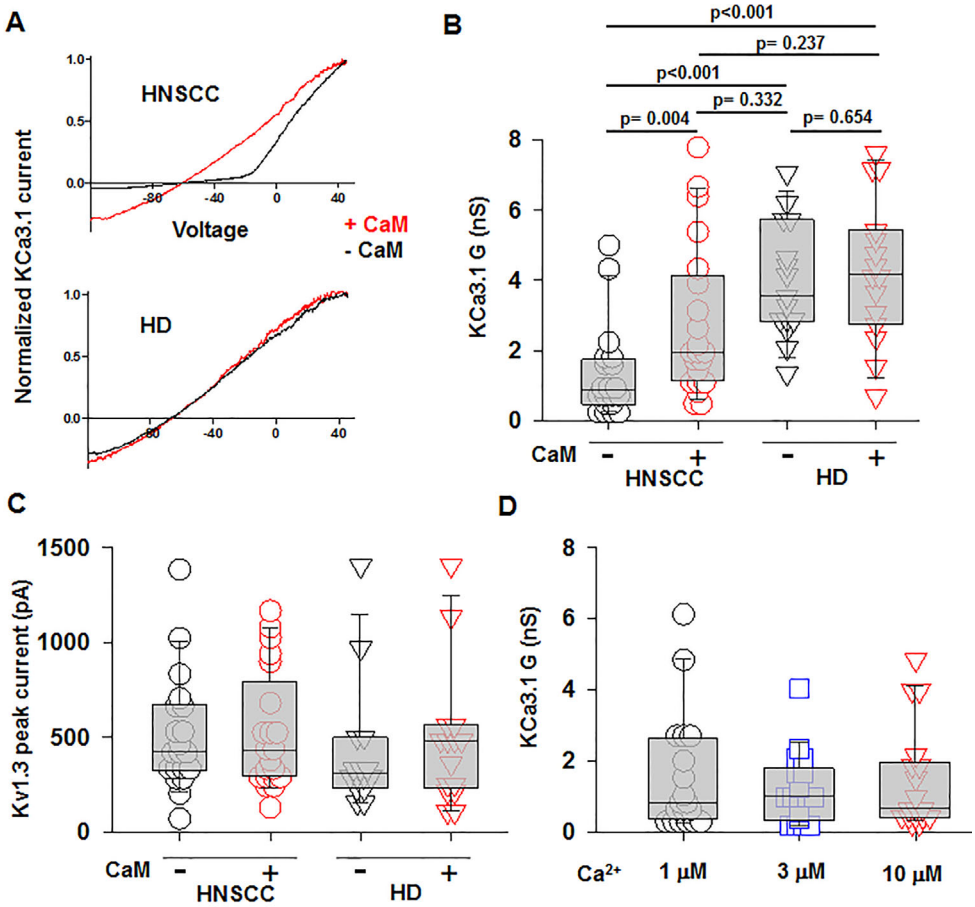


FIGURE 4 | Increased KCa3.1 activity in HNSCC T cells by intracellular delivery of CaM. **(A)** Representative KCa3.1 currents recorded in whole-cell voltage-clamp configuration in activated T cells from a HNSCC patient and a HD in the presence or absence of 50 µM CAM. The KCa3.1 currents are normalized for the maximum current at +50 mV to ease comparison of the KCa3.1 conductance at hyperpolarizing voltages. **(B)** KCa3.1 conductance, G (Panel **C**) and Kv1.3 peak currents measured in the presence or absence of 50 µM CaM in T cells from HNSCC patients (n = 20 cells without CaM and n = 21 cells with CaM from 4 patients) and HD (n = 20 cells from three individuals). **(D)** KCa3.1 G measured in the presence 1 µM, 3 µM, and 10 µM free Ca^{2+} in the patch pipette in activated HNSCC T cells (n = 18 cells for each conditions from three patients). The values in panels B, C, and D are presented as box and whisker plots. The data are reported as the median (horizontal line), first (top box), and third quartiles (bottom box). The KCa3.1 G or Kv1.3 peak currents were measured in at least five cells for each condition in an individual. Data in **(B, C)** were analyzed by two-way ANOVA [**(B)**: $p < 0.001$ for HD vs HNSCC and $p = 0.046$ for + CaM *vs.* –CaM; **(C)**: $p = 0.359$ for HD *vs.* HNSCC and $p = 0.673$ for + CaM *vs.* –CaM], while data in **(D)** were analyzed by one-way repeated measures ANOVA ($p = 0.796$).

transfected HD T cells were used as controls) using the methodology described previously (Chimote et al., 2018). We measured CXCL12-mediated 3D chemotaxis in si*CALM1* or scr-RNA-transfected HD T cells in the presence or absence of a concomitant chemokine and adenosine gradient. A representative experiment is shown in **Figure 6D**. Y-COM (*y*-coordinate of the center of mass, i.e., the *y*-coordinate of the averaged position the cells achieved at the end of the chemotaxis experiment; red triangles in **Figure 6D**) was used as a parameter to evaluate the chemotactic response. We observed that HD T cells transfected with scr-RNA migrated towards the chemokine even in the presence of adenosine (**Figure 6E**). However, when we knocked down *CALM1*, the transfected HD T cells lost their ability to chemotax in the presence of adenosine, as indicated by a decrease in their Y-COM (**Figure 6E**). We also found no alterations of IFNγ production in si*CALM1* transfected HD T cells (**Figure 6F**). Overall, these data indicate that the functional phenotype of HNSCC T cells is reproduced in HD T cells when CaM abundance is decreased (Chimote et al., 2018). Based on these findings, we tested whether, like in HNSCC T cells, KCa3.1 activators could be used to restore the channel's activity in si*CALM1*-transfected HD cells.

Activation of KCa3.1 Potentiates the Chemotaxis of *CALM1*-Knockdown HD T Cells in the Presence of Adenosine

We investigated whether positive modulation of KCa3.1 by 1-ethyl-2-benzimidazolinone (1-EBIO) reversed the functional

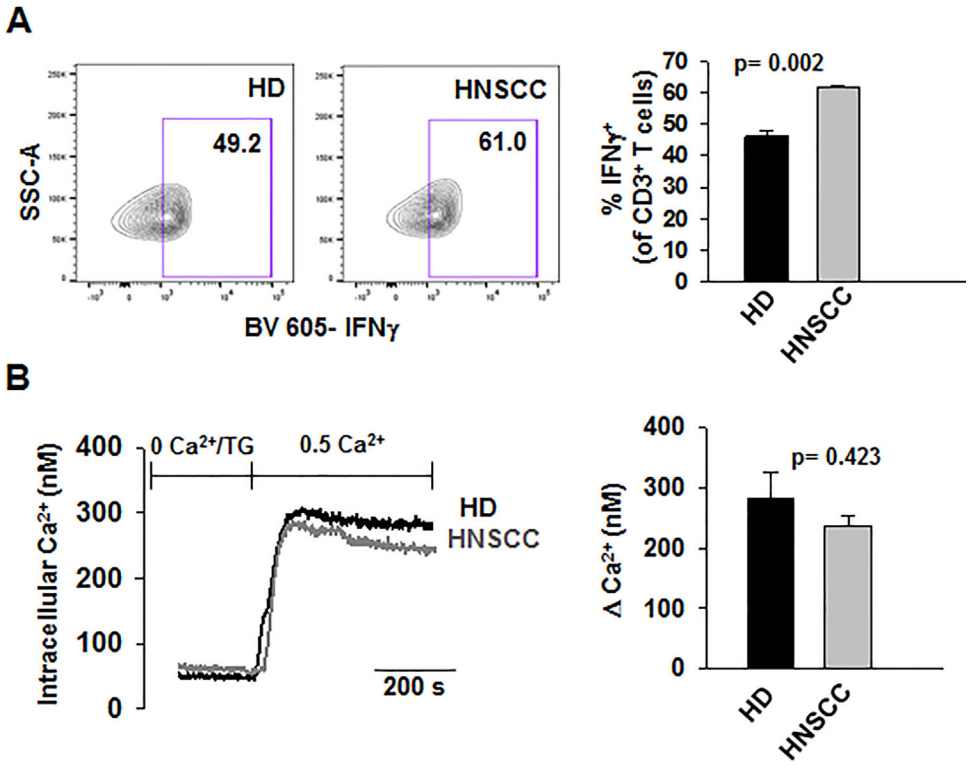


FIGURE 5 | Ca^{2+}- dependent T cell functions are not decreased in HNSCC T cells **(A)** Representative flow cytometry plots gated on stimulated $CD3^+$ T cells from a HD and HNSCC patient showing IFNγ production. (Right) Quantification of IFNγ-production in stimulated HD and HNSCC T cells. Cells were stimulated as described in *Materials and Methods*. Live cell population was identified by exclusion from Zombie Aqua live/dead stain and gated on $CD3^+$ T cell population. $IFN\gamma^+$ T cell populations were selected by drawing rectangle gates. The numbers within the gates are the percentages of $IFN\gamma^+$ $CD3^+$ T cells. **(B)** (Left) Representative Ca^{2+} response (presented as intracellular Ca^{2+} levels) recorded in a HD and HNSCC patient. Cells were loaded with Fura-2 and intracellular fluorescence was recorded and quantified using a standard curve as described in *Materials and Methods*. (Right) Average change in intracellular Ca^{2+}, expressed as ΔCa^{2+}, the difference between the recorded peak Ca^{2+} and baseline Ca^{2+} (see *Materials and Methods*) in HDs and HNSCC patients. In **(A, B)**, bars represent mean ± SEM from experiments performed in three HDs and HNSCC patients. Data were analyzed by Student's t-test.

consequences of *CALM1* knockdown in HD T cells (Coleman et al., 2014). We conducted chemotaxis experiments in si*CALM1*-transfected HD T cells in presence or absence of 1-EBIO. Consistent with our earlier observation (**Figure 6E**), the Y-COM values of si*CALM1* transfected HD T cells were significantly reduced in the presence of adenosine (**Figure 7A**). However, when the si*CALM1* transfected T cells from the same individual were preincubated with 1-EBIO, the Y-COM values in the presence of adenosine were almost threefold greater than those of cells that were not preincubated with 1-EBIO (**Figure 7A**). Thus, addition of 1-EBIO blocked the inhibitory effect of adenosine in HD T cells transfected with si*CALM1* (**Figure 7B**). These data indicate that enhancing the channel function by using KCa3.1 activators could be effective in correcting the chemotactic defects of HNSCC T cells. A question remains whether KCa3.1 activators could be of additional benefit in augmenting the anti-tumor functionality of HNSCC T cells. We therefore tested the effect of 1-EBIO on INFγ production. We observed that 1-EBIO significantly enhanced IFNγ production in both scr-RNA as well as si*CALM1*-transfected HD T cells (**Figure 7C**). The positive effect of 1-EBIO on IFNγ production was observed both in wild-type HNSCC as well as HD T cells (**Figure 7D**). Overall, these data indicate that potentiating the KCa3.1 function has a positive and broad impact on the functional capabilities of $CD8^+$ T cells of HNSCC patients.

DISCUSSION

The failure of immune surveillance in solid tumors has been ascribed, in part, to the inability of $CD8^+$ T cells to infiltrate the tumor and function in the TME. Circulating cytotoxic T cells of HNSCC patients display a reduced ability to chemotax in an adenosine-rich tumor-like microenvironment; a limitation arising from a decrease in their KCa3.1 channel activity (Chimote et al., 2018). In the present study, we provide evidence of a compartmentalized reduction in CaM in HNSCC T cells that compromises the activity of KCa3.1 and the chemotactic ability of these cells.

CaM is a Ca^{2+} sensor ubiquitously expressed in cells and required for multiple functions as it binds many signaling and structural proteins (Feske et al., 2003; Hogan et al., 2003; Berchtold and Villalobo, 2014; Adelman, 2016; Anguita and

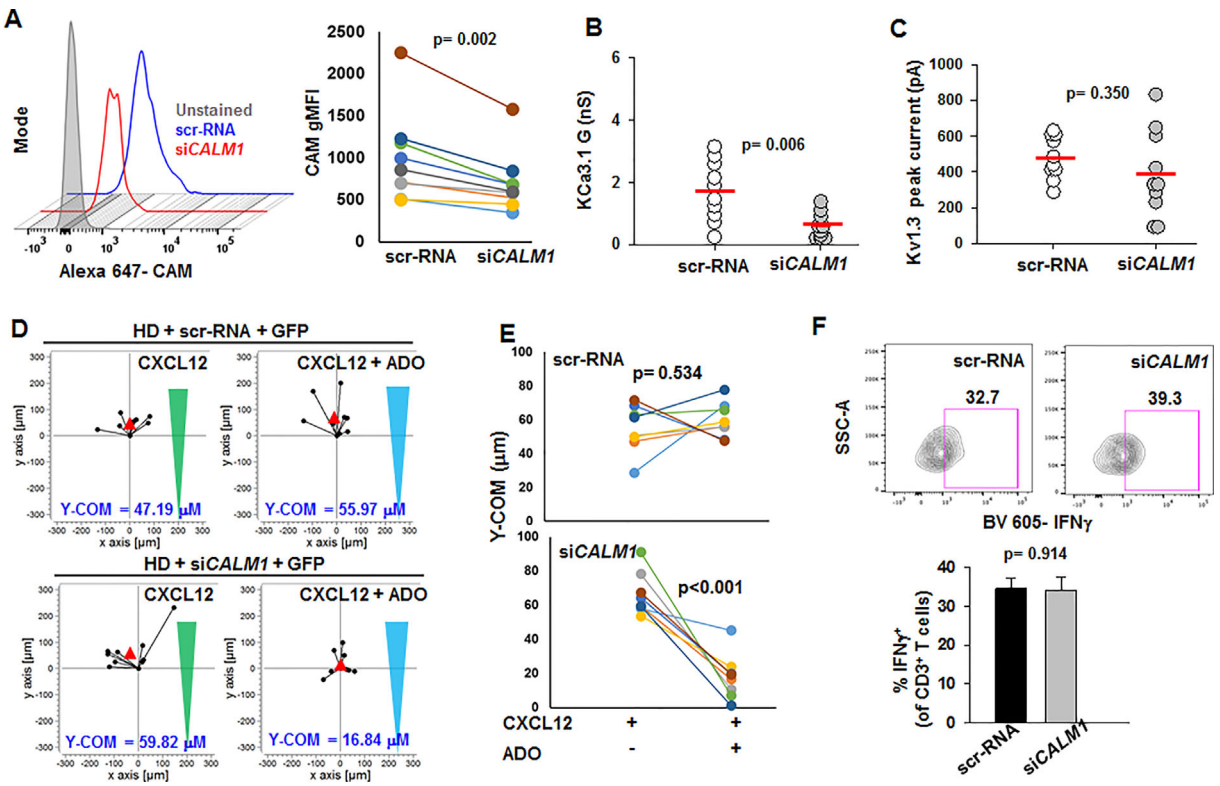


FIGURE 6 | Reduction of KCa3.1 activity and increased adenosine sensitivity in HD T cells by *CALM1* knockdown. (**A**, left) Representative flow cytometry overlay histogram showing CaM expression in activated T cells from one HD transfected with scr-RNA and si*CALM1*. (Right) gMFI for CAM expression in T cells from nine HD transfected with scr-RNA and si*CALM1*. Transfected cells were identified by positive staining for GFP. **(B, C)** KCa3.1 conductance **(B)** and Kv1.3 peak currents **(C)** in T cells transfected with either si*CALM1* (n = 10 cells) or scr-RNA (n = 10 cells) from two HDs. Transfected cells were identified by positive staining for GFP. Horizontal red line represents mean values for each group. **(D)** Trajectories of T cells co-transfected with GFP and either scr-RNA or si*CALM1* migrating along either a CXCL12 gradient (green triangles) or a combination gradient of CXCL12 and adenosine (blue triangles) in a representative HD. Trajectories of GFP positive cells (indicating successful transfection) are shown for each condition. The starting point for each trajectory is artificially set to the same origin. The red triangles represent Y-COM. **(E)** Y-COM values for cells migrating along either a CXCL12 gradient or a combination gradient of CXCL12 with adenosine in HD T cells transfected with scr-RNA (n = 8 donors) and si*CALM1* (n = 8 donors). **(F)** (Left) Representative flow cytometry plots gated on $CD3^+$ T cells transfected with either scr-RNA or si*CALM1* showing IFNγ production after stimulation. Live cell population was identified by exclusion from Zombie Aqua live/dead stain and gated on $CD3^+$ population and transfected cells were identified by positive staining for GFP. $IFN\gamma^+$ T cell populations were selected by drawing rectangle gates. The numbers next to the gates are the percentages of $IFN\gamma^+$ $CD3^+$ T GFP^+ cells. (Right) Quantification of IFNγ induction in scr-RNA or siCALM1 transfected $CD3^+$ T cells post 48 h activation in three HDs. Bars represent mean ± SEM. All data were analyzed by paired Student's t-test.

Villalobo, 2018). Herein we showed that HNSCC circulating T cells express less CaM than HD T cells. The lower abundance of CaM in HNSCC T cells was due to the combination of a decrease in *CALM1* and *CALM2* and the failure of these cells to upregulate CaM upon activation. CaM levels have been shown to increase with activation in healthy T cells (Colomer et al., 1993). A decrease in *CALM1* has been reported for $CD4^+$ TIL in breast cancer, and *CALM3* expression in TIL has been shown to be higher in extensively infiltrated tumors *vs* minimally infiltrated tumors suggesting a possible association between CaM levels and tumor infiltrating abilities of T cells (Gu-Trantien et al., 2013). Our data showed a plasma-membrane localized reduction of CaM in circulating T cells of HNSCC patients. There was no reduction in CaM in the rest of the cell. Indeed, we observed that the activity of KCa3.1 channels (a membrane-delimited CaM-dependent physiological process) of HNSCC T cells is affected by the reduction in membrane-proximal CaM, while other CaM-dependent functions like IFNγ production are still preserved.

KCa3.1 channels are Ca^{2+} dependent and require binding of intracellular Ca^{2+} to CaM, constitutively bound to the intracellular C-terminus of the channel, to open (Fanger et al., 1999; Khanna et al., 1999; Schumacher et al., 2001; Von Hahn et al., 2001; Stocker, 2004; Adelman, 2016; Lee and Mackinnon, 2018; Sforna et al., 2018). The importance of the CaM-KCa3.1 binding is underscored by the fact CaM co-immunoprecipitates with KCa3.1, and preventing the KCa3.1-CaM interaction by deletion of the C-terminus of KCa3.1, abrogates the channel function (Xia et al., 1998; Fanger et al., 1999; Khanna et al., 1999). Furthermore, addition of CaM antagonists prevents the binding of CaM to the channel and, consequently, reduces the KCa3.1 currents (Khanna et al., 1999; Von Hahn et al., 2001). This effect is reversed by addition of excess CaM intracellularly, suggesting that the KCa3.1-CaM interaction is

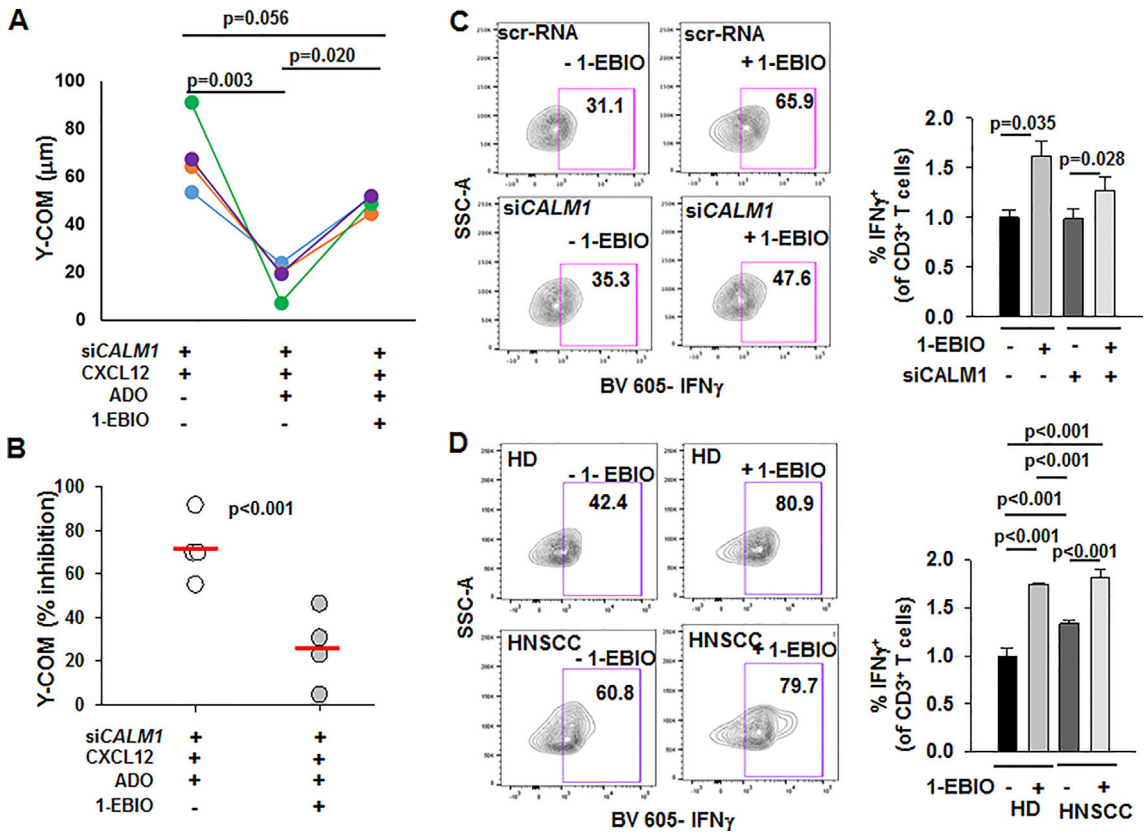


FIGURE 7 | Potentiation of chemotaxis in the presence of adenosine and INFγ production by 1-EBIO in *CALM1*-knockdown HD T cells. **(A)** Y-COM values calculated for HD T cells transfected with si*CALM1* migrating along either a CXCL12 gradient or a combination gradient of CXCL12 with adenosine (ADO) with or without preincubation with 20 µM 1-EBIO (n = 4 donors). Transfected cells, identified by positive GFP staining, were tracked for analyzing Y-COM. **(B)** Percentage inhibition by ADO of Y-COM **(B)** in cells pretreated with 1-EBIO. Horizontal red line represents mean values for each group. **(C)** (Left) Representative flow cytometry plots gated on $CD3^+$ T cells transfected with either scr-RNA or si*CALM1* with or without 1-EBIO (100 µM) showing IFNγ production after stimulation. Transfected cells were identified by positive staining for GFP. (Right) Quantification of IFNγ induction in scr-RNA or si*CALM1* transfected $CD3^+$ T cells with or without 1-EBIO in three HD. The data were normalized to IFNγ production in control scr-RNA transfected HD cells. Bars represent mean ± SEM. **(D)** (Left) Representative flow cytometry plots gated on HD or HNSCC $CD3^+$ T cells with or without 1-EBIO (100 µM) showing IFNγ production after stimulation. (Right) Quantification of IFNγ-producing HD and HNSCC T cells after stimulation in the presence or absence of 1-EBIO. The data were normalized to IFNγ production in control HD cells. For the HD + 1-EBIO condition, bars represent mean ± range for two individuals, while the rest of the bars in the graph represent mean ± SD for data from three HD and HNSCC patients. For analysis of **(C, D)**, live cell population was identified by exclusion from Zombie Aqua live/dead stain and gated on $CD3^+$ population and transfected cells were identified by positive staining for GFP (in panel **C**). $IFN\gamma^+$ T cell populations were selected by drawing rectangle gates. The numbers in the gates are the percentages of $IFN\gamma^+$ $CD3^+$ T cells. Data in **(A)** were analyzed with one-way repeated measures ANOVA ($p < 0.001$), in **(B)** with paired Student's t test, and in **(C)** with a two-way ANOVA [$p = 0.207$ for scr-RNA *vs.* si*CALM1* and $p = 0.006$ for – 1-EBIO vs. +1-EBIO]. Data in **(D)** were analyzed by Kruksal-Wallis One Way ANOVA on Ranks ($p < 0.001$).

reversible in nature (Khanna et al., 1999). While these studies have been conducted in heterologous expression systems, herein we are providing evidence that the KCa3.1-CaM interaction in $CD8^+$ T cells is disrupted in pathological conditions like HNSCC. Reduced KCa3.1-CaM interaction ultimately results in reduced channel function. Our PLA data (**Figure 3**) showed a 57% reduction in the association of CaM with KCa3.1 in the cell membrane of HNSCC T cells, which was comparable to the 67% inhibition of KCa3.1 currents measured in HNSCC T cells (**Figure 4**). This decrease in KCa3.1 activity observed in HNSCC *vs.* HD T cells was comparable to the reduction in HNSCC T cells previously described by us (Chimote et al., 2018). Furthermore, we observed that delivery of CaM intracellularly through the patch pipette increased KCa3.1, but not Kv1.3 currents in HNSCC T cells. It has been recently reported that CaM binds to the beta subunit of Kv1.3 channels in transfected HEK-293 cells, however this binding does not alter the function of the Kv1.3 channels (Solé et al., 2019). Our data also indicate that increasing the free Ca^{2+} concentration in the pipette did not increase the KCa3.1 activity in HNSCC T cells, thus indicating that CaM deficiency, and not a reduction in Ca^{2+} sensitivity due to other mechanisms such as changes in CaM and/or channel phosphorylation, is responsible for low KCa3.1 activity in HNSCC T cells (Fanger et al., 1999). The Ca^{2+} sensitivity of the channel is also modulated by kinases and phosphatases, like protein kinase A (PKA) and nucleoside diphosphate kinase B (NDPK-B) (Gerlach et al., 2001; Srivastava et al., 2005; Srivastava et al., 2006a; Srivastava et al.,

2006b; Srivastava et al., 2008; Srivastava et al., 2009; Di et al., 2010; Wong and Schlichter, 2014). Although we cannot exclude that alterations in these other pathways may exist in HNSCC T cells, our data clearly support a role for CaM reduction in HNSCC T cell dysfunction. In contrast to our findings in HNSCC T cells and to what was previously shown in other cell types, delivery of CaM intracellularly to HD T cells did not increase their KCa3.1 activity (Khanna et al., 1999; Joiner et al., 2001). It is possible that healthy T cell have sufficient amounts of intracellular CaM available to bind all the KCa3.1 channels on the membrane, which will leave the channel activity unchanged in presence of excess CaM. The contribution of CaM deficiency to the dysfunctional behavior of HNSCC T cells is further confirmed in HD T cells where knock-down of CaM conferred to these cells the same phenotype of HNSCC T cells. Silencing *CALM1* expression in HD T cells decreased KCa3.1 conductance (**Figure 6B**) to levels comparable to those of wild-type HNSCC T cells (**Figure 4B**) (Chimote et al., 2018). Furthermore, knock down of *CALM1* increased the sensitivity to adenosine of the HD T cells achieving an effect on chemotaxis comparable to that reported previously in HNSCC T cells (Chimote et al., 2018).

In accordance to what was previously shown in HNSCC T cells, our data showed that CaM deficiency reduces KCa3.1 activity, but not expression (Chimote et al., 2018). While it is well established that CaM regulates KCa3.1 activity, there are also reports that it facilitates the assembly of the pore-forming subunits and the surface trafficking of Ca^{2+}-activated K channels (Joiner et al., 2001; Lee et al., 2003). However, despite the decrease in CaM abundance in HNSCC T cells or when we knocked down CaM in HD T cells, we did not observe a reduction in KCa3.1 surface expression (Chimote et al., 2018). This could be attributed to a difference in the cell type studied as well as the fact that CaM is not solely responsible for the trafficking of the channel to the plasma membrane; leucine zipper domains on the C-terminus of KCa3.1 have been implicated as well (Khanna et al., 1999; Syme et al., 2003). This raises the possibility that even though when CaM levels are decreased, alternate pathways may still be able to effectively traffic the KCa3.1 channels to the cell membrane.

IFNγ production is also Ca^{2+}- and CaM-dependent. CaM regulates calcineurin activation, nuclear translocation of NFAT and production of downstream associated cytokines like IL-2 and IFNγ (Feske et al., 2003; Savignac et al., 2007; Liu, 2009). We did not observe a reduction in IFNγ production either in HNSCCC T cells or in *CALM1* transfected HD T cells. Instead, HNSCC T cells produced more IFNγ than HD T cells upon stimulation (**Figure 5A**). HNSCC and HD activated T cells display similar cytoplasmic CaM (**Figure 2C**), Ca^{2+} fluxing abilities (**Figure 5B**), and Kv1.3 channel activity (**Figure 4C**). The latter is sufficient to maintain the membrane hyperpolarization necessary for Ca^{2+} influx (Cahalan and Chandy, 2009). While these results can explain equal IFNγ production in HNSCC and HD T cells, the increase in IFNγ in HNSCC T cells may be due to enhanced TCR signaling upstream to ion channels. A robust increase in IFNγ in activated HNSCC lymphocytes (4-fold higher than in HD) was previously reported by others (Conti-Freitas et al., 2012).

Ultimately, herein we provide evidence that the negative functional consequences of CaM reduction in HNSCC T cells could be rescued by 1-EBIO. We have previously reported that activation of KCa3.1 channels by 1-EBIO or NS309 restored KCa3.1 function in HNSCC $CD8^+$ T cells and abrogated the inhibitory effect of adenosine (Chimote et al., 2018). Eil et al. have recently shown that 1-EBIO increased INFγ production in mouse T cells (Eil et al., 2016). In this study, we showed that KCa3.1 activators can be used as a modality to correct the chemotactic abnormalities associated to CaM deficiency in $CD8^+$ T cells of cancer patients. We also show that they can enhance INFγ production in both HNSCC and HD T cells indicating that this effect is independent of the CaM-deficiency and it is a consequence of the increased Ca^{2+} driving force that is achieved by activating KCa3.1 channels. This finding strengthens the therapeutic potentials of KCa3.1 activators, which could restore cytotoxic T cell functionality, and ultimately favor increased tumor infiltration and enhanced anti-tumor activity.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Cincinnati Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conception and design: AC, and LC. Development of methodology: AC, and LC. Acquisition of data: AC, VG, and HN. Provision and management of patients: TW-D. Provision of patient data: TW-D. Analysis and interpretation of data: AC, VG, HN, TW-D, and LC. Writing, review, and/or revision of the manuscript: AC and LC. Study supervision: LC. All authors discussed the results and commented on the manuscript.

FUNDING

This work was funded by grant support from the NIH (grant R01CA95286) to LC. HN received grant support from the NIH (T32CA117846T). TW-D was supported by a Clinical and Translational Science Award (CTSA)–awarded KL2 Mentored grant and a grant from CCTST (1UL1TR001425-01).

ACKNOWLEDGMENTS

We would like to acknowledge the clinical coordinators, specifically Ms. B. Fuhrman and Ms. S. Desai, from the University of Cincinnati Cancer Institute's Clinical Trials office (UCCI CTO) for their assistance in collection of patient samples. We thank Mr. E. Madis and Mr. M. G. Feria (Division of Nephrology and Hypertension, Department of Internal Medicine, University of Cincinnati) for technical assistance. We also thank Dr. H. Duncan (Division of Nephrology and Hypertension, Department of Internal Medicine, University of Cincinnati) for assistance with IRB regulatory affairs. We would like to thank Dr. R. Jandarov (Department of Biostatistics and Bioinformatics, University of Cincinnati) for valuable advice on statistical analysis. We would also like to thank the Center for Clinical and Translational Science and Training (CCTST), University of Cincinnati, for providing REDCap for effective patient data management. Confocal microscopy images were acquired at the Live Microscopy Core, Department of Pharmacology and Systems Physiology, University of Cincinnati. All flow cytometric data were acquired using equipment maintained by the Research Flow Cytometry Core in the Division of Rheumatology at Cincinnati Children's Hospital Medical Center and Shriners Hospital for Children Flow Cytometry Core, Cincinnati, OH.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00143/full#supplementary-material

SUPLEMENTARY TABLE S1 | Clinical and pathological characteristics of HNSCC patients.

SUPLEMENTARY TABLE S2 | CALM1 expression in HNSCC cells calculated with 18S rRNA and GAPDH as housekeeping genes.

SUPLEMENTARY TABLE S3 | Cell capacitance of activated T cells isolated from HD and HNSCC patients used in electrophysiological experiments.

SUPPLEMENTARY FIGURE 1 | Assessment of background staining for KCa3.1-biotin antibody.

SUPPLEMENTARY FIGURE 2 | Specificity of rabbit anti-human CaM antibody used for PLA experiments.

SUPPLEMENTARY FIGURE 3 | Technical controls for PLA.

SUPPLEMENTARY FIGURE 4 | KCa3.1 and CaM staining of HD and HNSCC T cells.

SUPPLEMENTARY FIGURE 5 | KCa3.1 expression and activation status of HD T cells transfected with siCALM1.

REFERENCES

Adelman, J. P. (2016). SK channels and calmodulin. *Channels* 10, 1–6. doi: 10.1080/19336950.2015.1029688

Anguita, E., and Villalobo, A. (2018). Ca^{2+} signaling and Src-kinases-controlled cellular functions. *Arch. Biochem. biophysics* 650, 59–74. doi: 10.1016/j.abb.2018.05.005

Balermpas, P., Rodel, F., Weiss, C., Rodel, C., and Fokas, E. (2014). Tumor-infiltrating lymphocytes favor the response to chemoradiotherapy of head and neck cancer. *Oncoimmunology* 3, e27403. doi: 10.4161/onci.27403

Berchtold, M. W., and Villalobo, A. (2014). The many faces of calmodulin in cell proliferation, programmed cell death, autophagy, and cancer. *Biochim. Biophys. Acta* 1843, 398–435. doi: 10.1016/j.bbamcr.2013.10.021

Cahalan, M. D., and Chandy, K. G. (2009). The functional network of ion channels in T lymphocytes. *Immunol. Rev.* 231, 59–87. doi: 10.1111/j.1600-065X.2009.00816.x

Chimote, A. A., Kuras, Z., and Conforti, L. (2012). Disruption of Kv1.3 channel forward vesicular trafficking by hypoxia in human T lymphocytes. *J. Biol. Chem.* 287, 2055–2067. doi: 10.1074/jbc.M111.274209

Chimote, A. A., Hajdu, P., Kucher, V., Boiko, N., Kuras, Z., Szilagyi, O., et al. (2013). Selective inhibition of KCa3.1 channels mediates adenosine regulation of the motility of human T cells. *J. Immunol.* 191, 6273–6280. doi: 10.4049/jimmunol.1300702

Chimote, A. A., Hajdu, P., Sfyris, A. M., Gleich, B. N., Wise-Draper, T., Casper, K. A., et al. (2017). Kv1.3 Channels mark functionally competent $cd8^+$ tumor-infiltrating lymphocytes in head and neck cancer. *Cancer Res.* 77, 53–61. doi: 10.1158/0008-5472.CAN-16-2372

Chimote, A. A., Balajthy, A., Arnold, M. J., Newton, H. S., Hajdu, P., Qualtieri, J., et al. (2018). A defect in KCa3.1 channel activity limits the ability of $CD8^+$ T cells from cancer patients to infiltrate an adenosine-rich microenvironment. *Sci. Signal.* 11, eaaq1616. doi: 10.1126/scisignal.aaq1616

Coleman, N., Brown, B. M., Oliván-Viguera, A., Singh, V., Olmstead, M. M., Valero, M. S., et al. (2014). New positive Ca^{2+}-activated K^+ channel gating modulators with selectivity for KCa3.1. *Mol. Pharmacol.* 86, 342–357. doi: 10.1124/mol.114.093286

Colomer, J., Agell, N., Engel, P., Alberola-Ila, J., and Bachs, O. (1993). Calmodulin expression during proliferative activation of human T lymphocytes. *Cell Calcium* 14, 609–618. doi: 10.1016/0143-4160(93)90061-A

Conti-Freitas, L. C., Foss-Freitas, M. C., Mamede, R. C., and Foss, N. T. (2012). Interferon-gamma and interleukin-10 production by mononuclear cells from patients with advanced head and neck cancer. *Clinics* 67, 587–590. doi: 10.6061/clinics/2012(06)07

Di, L., Srivastava, S., Zhdanova, O., Sun, Y., Li, Z., and Skolnik, E. Y. (2010). Nucleoside diphosphate kinase B knock-out mice have impaired activation of the K^+ channel KCa3.1, resulting in defective T cell activation. *J. Biol. Chem.* 285, 38765–38771. doi: 10.1074/jbc.M110.168070

Eil, R., Vodnala, S. K., Clever, D., Klebanoff, C. A., Sukumar, M., Pan, J. H., et al. (2016). Ionic immune suppression within the tumour microenvironment limits T cell effector function. *Nature* 537, 539–543. doi: 10.1038/nature19364

Fang, J., Li, X., Ma, D., Liu, X., Chen, Y., Wang, Y., et al. (2017). Prognostic significance of tumor infiltrating immune cells in oral squamous cell carcinoma. *BMC Cancer* 17, 375. doi: 10.1186/s12885-017-3317-2

Fanger, C. M., Ghanshani, S., Logsdon, N. J., Rauer, H., Kalman, K., Zhou, J., et al. (1999). Calmodulin mediates calcium-dependent activation of the intermediate conductance KCa channel, IKCa1. *J. Biol. Chem.* 274, 5746–5754. doi: 10.1074/jbc.274.9.5746

Feske, S., Okamura, H., Hogan, P. G., and Rao, A. (2003). Ca^{2+}/calcineurin signalling in cells of the immune system. *Biochem. Biophys. Res. Commun.* 311, 1117–1132. doi: 10.1016/j.bbrc.2003.09.174

Feske, S., Skolnik, E. Y., and Prakriya, M. (2012). Ion channels and transporters in lymphocyte function and immunity. *Nat. Rev. Immunol.* 12, 532–547. doi: 10.1038/nri3233

Gerlach, A. C., Syme, C. A., Giltinan, L., Adelman, J. P., and Devors, D. C. (2001). ATP-dependent activation of the intermediate conductance, Ca^{2+}-activated K^+ channel, hIK1, is conferred by a C-terminal domain. *J. Biol. Chem.* 276, 10963–10970. doi: 10.1074/jbc.M007716200

Ghanshani, S., Wulff, H., Miller, M. J., Rohm, H., Neben, A., Gutman, G. A., et al. (2000). Up-regulation of the ikca1 potassium channel during t-cell activation: molecular mechanism and functional consequences. *J. Biol. Chem.* 275, 37137–37149. doi: 10.1074/jbc.M003941200

Gurusamy, D., Clever, D., Eil, R., and Restifo, N. P. (2017). Novel "Elements". *Immune Suppression within Tumor Microenvironment. Cancer Immunol. Res.* 5, 426–433. doi: 10.1158/2326-6066.CIR-17-0117

Gu-Trantien, C., Loi, S., Garaud, S., Equeter, C., Libin, M., De Wind, A., et al. (2013). $CD4^+$ follicular helper T cell infiltration predicts breast cancer survival. *J. Clin. Invest.* 123, 2873–2892. doi: 10.1172/JCI67428

Hajdu, P., Martin, G. V., Chimote, A. A., Szilagyi, O., Takimoto, K., and Conforti, L. (2015). The C-terminus SH3-binding domain of Kv1.3 is required for the actin-mediated immobilization of the channel *via* cortactin. *Mol. Biol. Cell* 26, 1640–1651. doi: 10.1091/mbc.E14-07-1195

Hogan, P. G., Chen, L., Nardone, J., and Rao, A. (2003). Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev.* 17, 2205–2232. doi: 10.1101/gad.1102703

Joiner, W. J., Khanna, R., Schlichter, L. C., and Kaczmarek, L. K. (2001). Calmodulin regulates assembly and trafficking of SK4/IK1 Ca^{2+}-activated K^+ channels. *J. Biol. Chem.* 276, 37980–37985. doi: 10.1074/jbc.M104965200

Joyce, J. A., and Fearon, D. T. (2015). T cell exclusion, immune privilege, and the tumor microenvironment. *Science* 348, 74–80. doi: 10.1126/science.aaa6204

Khanna, R., Chang, M. C., Joiner, W. J., Kaczmarek, L. K., and Schlichter, L. C. (1999). hSK4/hIK1, a calmodulin-binding KCa channel in human T lymphocytes. Roles in proliferation and volume regulation. *J. Biol. Chem.* 274, 14838–14849. doi: 10.1074/jbc.274.21.14838

Kuras, Z., Yun, Y. H., Chimote, A. A., Neumeier, L., and Conforti, L. (2012). KCa3.1 and TRPM7 channels at the uropod regulate migration of activated human T cells. *PloS One* 7, e43859. doi: 10.1371/journal.pone.0043859

Lee, C. H., and Mackinnon, R. (2018). Activation mechanism of a human SK-calmodulin channel complex elucidated by cryo-EM structures. *Science* 360, 508–513. doi: 10.1126/science.aas9466

Lee, W. S., Ngo-Anh, T. J., Bruening-Wright, A., Maylie, J., and Adelman, J. P. (2003). Small conductance Ca^{2+}-activated K^+ channels and calmodulin: cell surface expression and gating. *J. Biol. Chem.* 278, 25940–25946. doi: 10.1074/jbc.M302091200

Liu, J. O. (2009). Calmodulin-dependent phosphatase, kinases, and transcriptional corepressors involved in T-cell activation. *Immunol. Rev.* 228, 184–198. doi: 10.1111/j.1600-065X.2008.00756.x

Mandapathil, M., Szczepanski, M., Harasymczuk, M., Ren, J., Cheng, D., Jackson, E. K., et al. (2012). CD26 expression and adenosine deaminase activity in regulatory T cells (Treg) and $CD4^+$ T effector cells in patients with head and neck squamous cell carcinoma. *Oncoimmunology* 1, 659–669. doi: 10.4161/onci.20387

Marshall, C. B., Nishikawa, T., Osawa, M., Stathopulos, P. B., and Ikura, M. (2015). Calmodulin and STIM proteins: Two major calcium sensors in the cytoplasm and endoplasmic reticulum. *Biochem. Biophys. Res. Commun.* 460, 5–21. doi: 10.1016/j.bbrc.2015.01.106

Robbins, J. R., Lee, S. M., Filipovich, A. H., Szigligeti, P., Neumeier, L., Petrovic, M., et al. (2005). Hypoxia modulates early events in T cell receptor-mediated activation in human T lymphocytes *via* Kv1.3 channels. *J. Physiol.* 564, 131–143. doi: 10.1113/jphysiol.2004.081893

Savignac, M., Mellstrom, B., and Naranjo, J. R. (2007). Calcium-dependent transcription of cytokine genes in T lymphocytes. *Pflugers Archiv: Eur. J. Physiol.* 454, 523–533. doi: 10.1007/s00424-007-0238-y

Schumacher, M. A., Rivard, A. F., Bachinger, H. P., and Adelman, J. P. (2001). Structure of the gating domain of a Ca^{2+}-activated K^+ channel complexed with Ca2+/calmodulin. *Nature* 410, 1120–1124. doi: 10.1038/35074145

Sforna, L., Megaro, A., Pessia, M., Franciolini, F., and Catacuzzeno, L. (2018). Structure, gating and basic functions of the ca^{2+}-activated k channel of intermediate conductance. *Curr. Neuropharmacol.* 16, 608–617. doi: 10.2174/1570159X15666170830122402

Solé, L., Roig, S. R., Sastre, D., Vallejo-Gracia, A., Serrano-Albarrás, A., Ferrer-Montiel, A., et al. (2019). The calmodulin-binding tetraleucine motif of KCNE4 is responsible for association with Kv1.3. *FASEB J.* 33, 8263–8279. doi: 10.1096/fj.201801164RR

Srivastava, S., Li, Z., Lin, L., Liu, G., Ko, K., Coetzee, W. A., et al. (2005). The phosphatidylinositol 3-phosphate phosphatase myotubularin- related protein 6 (MTMR6) is a negative regulator of the Ca^{2+}-activated K^+ channel KCa3.1. *Mol. Cell. Biol.* 25, 3630–3638. doi: 10.1128/MCB.25.9.3630-3638.2005

Srivastava, S., Choudhury, P., Li, Z., Liu, G., Nadkarni, V., Ko, K., et al. (2006a). Phosphatidylinositol 3-phosphate indirectly activates KCa3.1 *via* 14 amino acids in the carboxy terminus of KCa3.1. *Mol. Biol. Cell* 17, 146–154. doi: 10.1091/mbc.e05-08-0763

Srivastava, S., Li, Z., Ko, K., Choudhury, P., Albaqumi, M., Johnson, A. K., et al. (2006b). Histidine phosphorylation of the potassium channel KCa3.1 by nucleoside diphosphate kinase B is required for activation of KCa3.1 and CD4 T cells. *Mol. Cell* 24, 665–675. doi: 10.1016/j.molcel.2006.11.012

Srivastava, S., Zhdanova, O., Di, L., Li, Z., Albaqumi, M., Wulff, H., et al. (2008). Protein histidine phosphatase 1 negatively regulates CD4 T cells by inhibiting the K^+ channel KCa3.1. *Proc. Natl. Acad. Sci. U. S. A.* 105, 14442–14446. doi: 10.1073/pnas.0803678105

Srivastava, S., Di, L., Zhdanova, O., Li, Z., Vardhana, S., Wan, Q., et al. (2009). The class II phosphatidylinositol 3 kinase C2beta is required for the activation of the K^+ channel KCa3.1 and CD4 T-cells. *Mol. Biol. Cell* 20, 3783–3791. doi: 10.1091/mbc.e09-05-0390

Stocker, M. (2004). Ca^{2+}-activated K^+ channels: molecular determinants and function of the SK family. *Nat. Rev. Neurosci.* 5, 758–770. doi: 10.1038/nrn1516

Syme, C. A., Hamilton, K. L., Jones, H. M., Gerlach, A. C., Giltinan, L., Papworth, G. D., et al. (2003). Trafficking of the Ca^{2+}-activated K^+ channel, hIK1, is dependent upon a C-terminal leucine zipper. *J. Biol. Chem.* 278, 8476–8486. doi: 10.1074/jbc.M210072200

Uppaluri, R., Dunn, G. P., and Lewis, J. S. (2008). Focus on TILs: prognostic significance of tumor infiltrating lymphocytes in head and neck cancers. *Cancer Immun.* 8, 16.

Von Hahn, T., Thiele, I., Zingaro, L., Hamm, K., Garcia-Alzamora, M., Köttgen, M., et al. (2001). Characterisation of the Rat SK4/IK1 K^+ Channel. *Cell. Physiol. Biochem.* 11, 219–230. doi: 10.1159/000051936

Wong, R., and Schlichter, L. C. (2014). PKA reduces the rat and human KCa3.1 current, CaM binding, and Ca^{2+} signaling, which requires Ser332/334 in the CaM-binding C terminus. *J. Neurosci.* 34, 13371–13383. doi: 10.1523/JNEUROSCI.1008-14.2014

Xia, X. M., Fakler, B., Rivard, A., Wayman, G., Johnson-Pais, T., Keen, J. E., et al. (1998). Mechanism of calcium gating in small-conductance calcium-activated potassium channels. *Nature* 395, 503–507. doi: 10.1038/26758

Zhong, L. P., Zhang, C. P., Ren, G. X., Guo, W., William, W. N., Sun, J., et al. (2013). Randomized phase III trial of induction chemotherapy with docetaxel, cisplatin, and fluorouracil followed by surgery versus up-front surgery in locally advanced resectable oral squamous cell carcinoma. *J. Clin. Oncol.* 31, 744–751. doi: 10.1200/JCO.2012.43.8820

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 Chimote, Gawali, Newton, Wise-Draper and Conforti. 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 Pharmacology

ORIGINAL RESEARCH
published: 24 April 2020
doi: 10.3389/fphar.2020.00489

hERG K^+ Channels Promote Survival of Irradiated Leukemia Cells

Daniela Palme[1†], Milan Misovic[1†], Katrin Ganser[1], Lukas Klumpp[1], Helmut R. Salih[2], Daniel Zips[1,3,4] and Stephan M. Huber[1]*

[1] Department of Radiation Oncology, University Hospital Tübingen, Tübingen, Germany, [2] Clinical Collaboration Unit Translational Immunology, German Cancer Consortium (DKTK), University Hospital Tübingen, Tübingen, Germany, [3] German Cancer Consortium (DKTK), Partner Site Tübingen, Tübingen, Germany, [4] German Cancer Research Center (DKFZ), Heidelberg, Germany

OPEN ACCESS

Edited by:
Sébastien Roger,
Université de Tours, France

Reviewed by:
Luis A. Pardo,
Max-Planck-Gesellschaft (MPG), Germany
Jacques Barhanin,
University of Nice Sophia Antipolis, France

***Correspondence:**
Stephan M. Huber
stephan.huber@uni-tuebingen.de

†Present addresses:
Daniela Palme,
AstraZeneca GmbH,
Wedel, Germany
Milan Misovic,
Department of Gastroenterology,
Klinikum Bayreuth GmbH,
Bayreuth, Germany

Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: 09 December 2019
Accepted: 27 March 2020
Published: 24 April 2020

Citation:
Palme D, Misovic M, Ganser K, Klumpp L, Salih HR, Zips D and Huber SM (2020) hERG K^+ Channels Promote Survival of Irradiated Leukemia Cells. Front. Pharmacol. 11:489. doi: 10.3389/fphar.2020.00489

Many tumor cells express highly elevated activities of voltage-gated K^+ channels in the plasma membrane which are indispensable for tumor growth. To test for K^+ channel function during DNA damage response, we subjected human chronic myeloid leukemia (CML) cells to sub-lethal doses of ionizing radiation (0–8 Gy, 6 MV photons) and determined K^+ channel activity, K^+ channel-dependent Ca^{2+} signaling, cell cycle progression, DNA repair, and clonogenic survival by whole-cell patch clamp recording, fura-2 Ca^{2+} imaging, Western blotting, flow cytometry, immunofluorescence microscopy, and pre-plating colony formation assay, respectively. As a result, the human erythroid CML cell line K562 and primary human CML cells functionally expressed hERG1. Irradiation stimulated in both cell types an increase in the activity of hERG1 K^+ channels which became apparent 1–2 h post-irradiation. This increase in K^+ channel activity was paralleled by an accumulation in S phase of cell cycle followed by a G_2/M cell cycle arrest as analyzed between 8 and 72 h post-irradiation. Attenuating the K^+ channel function by applying the hERG1 channel inhibitor E4031 modulated Ca^{2+} signaling, impaired inhibition of the mitosis promoting subunit cdc2, overrode cell cycle arrest, and decreased clonogenic survival of the irradiated cells but did not affect repair of DNA double strand breaks suggesting a critical role of the hERG1 K^+ channels for the Ca^{2+} signaling and the cell cycle control during DNA damage response.

Keywords: ionizing radiation, patch-clamp whole-cell recording, flow cytometry, hERG1 potassium channels, S progression, G_2/M arrest

INTRODUCTION

Tumor cells express ion channel toolkits that differ from that of their healthy parental counterparts. Importantly, this altered ion channel expression serves pivotal functions in neoplastic transformation, survival, proliferation, migration, invasion and metastasis, or therapy resistance of tumor cells suggesting ion channels as potential targets in antitumor therapy. Notably, certain individual types of ion channels are overexpressed in several different tumor entities classifying them as channel with high oncogenic function [for review see (Huber, 2013)]. Among those are ether-à-go-go-related (hERG1, K_v11.1, *KCNH2*) voltage-gated, fast inactivating human K^+ channels (Vandenberg et al., 2012) that have been reported in several tumor entities including chronic

myeloid leukemia (CML) cells (Arcangeli, 2005). Non-neoplastic bone marrow $CD34^+/CD38^-$ hematopoietic stem cells (Li et al., 2008) and resting or proliferating lymphocytes (Smith et al., 2002), in contrast, do not express hERG1.

hERG1 has been demonstrated predominantly by the seminal work of Annarosa Arcangeli's group in Florence, Italy, to accomplish outside-in signaling in adhesomes that are macromolecular signaling complexes at focal adhesion sites. hERG1 channels physically interact with receptors in the plasma membrane such as β1-integrin [for review see (Becchetti et al., 2019)]. Functionally, hERG1 may contribute to survival, proliferation (Wang et al., 2002), and metastasis of tumor cells (Manoli et al., 2019). Moreover, hERG1 reportedly may promote tumor vascularization (Crociani et al., 2013) and confer resistance against chemotherapeutics (Pillozzi et al., 2011).

The vast majority of CML carries the Philadelphia chromosome that results from a reciprocal translocation of chromosomes 9 and 22. This translocation gives rise to the Bcr-Abl (breakpoint cluster region/Abelson) fusion oncogene which encodes a constitutively active tyrosine kinase, and tyrosine kinase inhibitors (TKI) such as imatinib are the first line therapy for CML. For a selected patient group (e.g., with TKI resistant tumors), however, hematopoietic stem cell transplantation after myeloablative conditioning remains a curative therapy option. Conditioning may involve total body irradiation and disease relapse is together with transplant toxicity the major cause of transplant failure in patients allografted for CML (Craddock, 2018). Notably, ionizing radiation has been demonstrated to activate K^+ channels which in turn contribute to DNA damage response and survival of the irradiated tumor cells [for review see (Huber et al., 2013)].

In particular, our previous work has demonstrated $K_v3.4$ K^+ channel activity in CML cells which is stimulated by ionizing radiation and which contributes to DNA damage response and survival of these cells (Palme et al., 2013). Given the upregulation of hERG1 in leukemia (see above), the present study aimed to test for a function of hERG1 channels in the stress response of leukemia cells treated with ionizing radiation. In addition, the crosstalk of $K_v3.4$ K^+ and hERG1 channels in irradiated CML cells should be characterized.

To this end, K562 CML cells and—in further experiments—primary CML cells were used as *in vitro* models since K562 cells reportedly express hERG1 (Smith et al., 2002) and respond to ionizing radiation with elevated $K_v3.4$ (Palme et al., 2013) and other plasmalemmal ion channel activity and Ca^{2+} signaling (Heise et al., 2010). The present study applied patch-clamp fast whole cell recording, fura-2 Ca^{2+} imaging, immunoblotting, flow cytometry, immunofluorescence microscopy, and colony formation assay to analyse radiogenic hERG1 activation, hERG1-dependent Ca^{2+} signaling and activation of Ca^{2+} effector proteins, bromodeoxyuridine (BrdU) incorporation and cell cycle progression, repair of DNA double-strand breaks, as well as cell death and clonogenic survival in irradiated CML cells.

MATERIAL AND METHODS

Cell Culture

Primary CML cells were isolated by density gradient centrifugation after obtaining informed consent in accordance with the Helsinki protocol, and the study was performed according to the guidelines of the local ethics committee. Primary CML cells and K562 human erythroid CML cells were cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium containing L-glutamine (Gibco, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS) and penicillin (100 U/ml)/streptomycin (100 µg/ml). Ionizing radiation (6 MV photons, single dose of 1–8 Gy) was applied by using a linear accelerator (LINAC SL25 Philips) at a dose rate of 4 Gy/min at room temperature. Following irradiation, cells were post-incubated in supplemented RPMI 1640 medium for 1–72 h (immunoblotting, patch-clamp, fura-2 Ca^{2+}-imaging, flow cytometry) and 2 weeks (colony formation).

Blockage of hERG1 and $K_v3.4$

According to a meta study (Polak et al., 2009) reported IC_{50} values for the blockage of hERG1 by the class III antiarrhythmic agent E4031 in expression systems *in vitro* range from 8 to 570 nM (mean 81 nM, median 17 nM, n = 14) which suggests a quantitative channel inhibition at a concentration around 200–800 nM in serum-free buffer solution. To compensate for binding to plasma proteins (Webster et al., 2001) and time-dependent drug degradation we applied in initial experiments 3 µM E4031, later on, we reduced to 1 µM. E4031 was initially dissolved in DMSO (< 0.1% DMSO final concentration). Further batches were dissolved in $_{dd}$H20. E4031-DMSO control, vehicle (DMSO), was added at the same concentration. To the best of our knowledge, E4031 at the applied concentration does not interfere with the non-hERG1 channels detected in K562 cells. Tetraethylammonium (TEA) which was used at a concentration of 3 mM to inhibit $K_v3.4$ channels does not exert relevant blockage of hERG1 channels [hERG1 IC_{50} = 50 mM TEA (Choi et al., 2011)]. For 3 mM TEA-containing NaCl solution (see below), 3 mM NaCl was replaced isosmotically by diluting 150 mM TEA solution with NaCl solution (see below) by a factor of 1:50.

Patch-Clamp Recording

K562 and primary CML cells were irradiated with 0 or 5 Gy. 1–4 h post irradiation, fast hERG1-mediated deactivating whole-cell tail currents were evoked by voltage square pulses delivered from different holding potentials/pre-pulses to voltages of −80 mV or −100 mV as indicated in the inserts of **Figures 1A**, **6A**. Currents were recorded (10 kHz sampling rate) and 3-kHz low-pass-filtered by an EPC-9 amplifier (HEKA, Lambrecht, Germany) using Pulse software (HEKA) and an ITC-16 Interface (InstruTech, Port Washington, NY, USA). Borosilicate glass pipettes (~5 MΩ pipette resistance; GC150 TF-10, Clark Medical Instruments, Pangbourne, UK) manufactured by a microprocessor-driven DMZ puller (Zeitz, Augsburg,

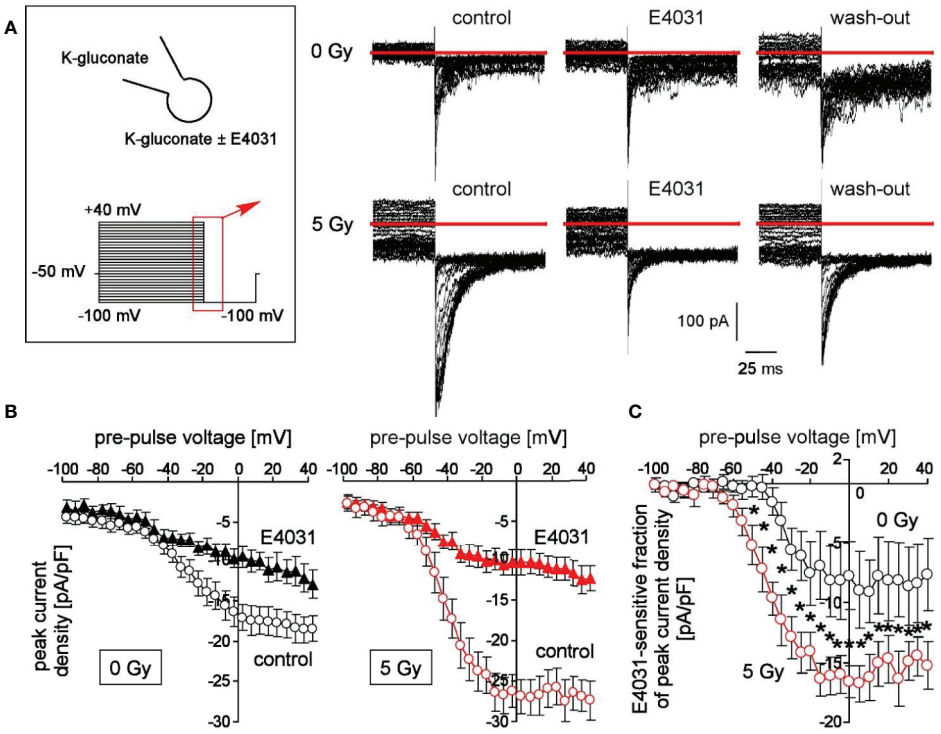


FIGURE 1 | Irradiation modifies voltage-dependent hERG channels in K562 cells. **(A)** Current tracings recorded from a control (0 Gy, upper line) and a 5 Gy-irradiated cell (lower line). Records were obtained with K-D-gluconate pipette and bath solution during a hyperpolarizing voltage step to −100 mV delivered from pre-pulses ranging from −100 to +100 mV (5 mV increments, see insert). Deactivating inward tail currents were recorded before (control), during (E4031) and after (wash-out) of the hERG1 inhibitor E4031 (1 μM). Red line indicates zero current. **(B)** Relationships between mean (± SE, n = 9–16) peak current density of the deactivating tail currents at −100 mV clamp voltage and the pre-pulse voltage in control (0 Gy, left) and 5 Gy-irradiated (right) K562 cells recorded as in **(A)** before (open circles) and during administration of E4031 (closed triangles). **(C)** Relationships between mean (± SE, n = 6–9) E4031-sensitive fractions of peak current density and activating pre-pulses in control (0 Gy, black circles) and 5 Gy-irradiated (red circles) as recorded in **(A)**. * indicates $p \leq 0.05$, two-tailed (Welch-corrected) t-test.

Germany) were used in combination with a scanning tunneling microscope (STM) electrical micromanipulator (Lang GmbH and Co KG, Germany).

Cells were continuously superfused at 37°C temperature with NaCl solution [in mM: 125 NaCl, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 KCl, 5 D-glucose, 1 $MgCl_2$, 1 $CaCl_2$, 0 or 0.001 E4031, titrated with NaOH to pH 7.4]. Superfusion (~1 ml/min) was applied through a heated flow system inserted into the dish (bath volume approximately 200 μl). The bath was grounded *via* a bridge filled with NaCl bath solution). Upon GΩ-seal formation (10–100 GΩ seal resistance) and entry into the whole-cell recording mode, cells were recorded with K-D-gluconate bath solution (in mM: 150 K-D-gluconate, 10 HEPES, 1 Ca-D-gluconate$_2$, titrated with KOH to pH 7.4) and K-D-gluconate pipette solution (in mM: 140 K-D-gluconate, 5 HEPES, 5 $MgCl_2$, 1 K_2-ethylene glycol-bis(-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 K_2-ATP, titrated with KOH to pH 7.4).

Clamp voltages refer to the cytosolic face of the plasma membrane and were corrected offline for the applying liquid junction potential according to Barry and Lynch (Barry and Lynch, 1991). In particular, for calculation of the liquid junction potentials relative mobilities of 1.0388, 0.33, 0.3, 1.0, 0.682, 0.488, and 0.361 were assumed for chloride, gluconate, HEPES, potassium, calcium, sodium, and magnesium, respectively. The set-off liquid junction potential (as measured against the earthed bridge connected to the bath both containing NaCl solution) between K-D-gluconate pipette- and NaCl bath solution was +14.9 mV which applies with −14.9 mV at the intracellular plasma membrane face upon giga seal formation and entry into the whole-cell mode. The liquid junction potential between K-D-gluconate bath solution and NaCl bridge applying at the extracellular membrane face upon bath exchange was calculated to −14.6 mV suggesting an effective deviation from the adjusted clamp voltage of −0.3 mV. Inward currents are defined as influx of cations into the cells (or efflux of anions out of the cell), depicted as downward deflections of the current tracings, and defined as negative currents in the current voltage relationships. Peak currents were analyzed.

Fura-2 Ca^{2+} Imaging

Fluorescence measurements were performed by the use of an inverted phase-contrast microscope (Axiovert 100; Zeiss, Oberkochen, Germany). Fluorescence was evoked by a filter wheel (Visitron Systems, Puchheim, Germany)-mediated alternative excitation at 340/26 or 387/11 nm (AHF, Analysentechnik, Tübingen, Germany). Excitation and emission

light were deflected by a dichroic mirror (409/LP nm beamsplitter, AHF) into the objective (Fluar x40/1.30 oil; Zeiss) and transmitted to the camera (Visitron Systems), respectively. Emitted fluorescence intensity was recorded at 587/35 nm (AHF). Excitation was controlled and data acquired by Metafluor computer software (Universal Imaging, Downingtown, PA, USA). The 340/380-nm fluorescence ratio was used as a measure of cytosolic free Ca^{2+} concentration ($c[Ca^{2+}]_{free}$). K562 cells were irradiated (0 or 5 Gy) and loaded with fura-2/AM (2 μM for 30 min at 37°C; Molecular Probes, Göttingen, Germany) in supplemented RPMI medium. Steady state $c[Ca^{2+}]_{free}$ was recorded in irradiated (0 or 5 Gy) K562 cells (1–4 h post-irradiation) in the continuous presence of external Ca^{2+} during superfusion with Ca^{2+}-containing NaCl solution (see above) before, during, and after administration of E4013 (1μM) or TEA (3 mM).

Western Blotting

Irradiated K562 cells (0 and 5 Gy, 2 h post-radiation) were lysed in a buffer (containing in mM: 50 HEPES, pH 7.5, 150 NaCl, 1 ethylenediaminetetraacetic acid (EDTA), 10 sodium pyrophosphate, 10 NaF, 2 Na_3VO_4, 1 phenylmethylsulfonylfluorid (PMSF) additionally containing 1% Triton X-100, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 3 μg/ml pepstatin) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition. In some experiments cells were pre-incubated (0.5 h), irradiated and post-incubated (2 h) in the presence of the hERG1 channel inhibitor E4013 (0 or 1 μM). SDS-PAGE-segregated proteins were electro-transferred onto PVDF membranes (Roth, Karlsruhe, Germany). Blots were blocked in Tris-buffered saline (TBS) buffer containing 0.05% Tween 20 and 5% non-fat dry milk for 1 h at room temperature. The membrane was incubated overnight at 4°C with the following primary antibodies (in TBS-Tween / 5% milk): rabbit anti-phospho-CaMKII (Thr286) antibody (Cell Signaling #3361, New England Biolabs, Frankfurt, Germany, 1:1,000), rabbit anti-CaMKII (pan) antibody (Cell Signaling #3362, 1:1,000), rabbit anti-phospho-cdc2 (Tyr15) (Cell Signaling #9111, 1:1,000), or β-actin (mouse anti-β-actin antibody, clone AC-74, Sigma #A2228 1:20,000). Antibody binding was detected with a horseradish peroxidase-linked goat anti-rabbit IgG antibody or anti-mouse IgG antibody (Cell Signaling # 7074 and #7076, respectively; 1:1,000–1:2,000 dilution in TBS-Tween/5% milk) incubated for 1 h at room temperature and enhanced chemiluminescence (ECL Western blotting analysis system, GE Healthcare/Amersham-Biosciences, Freiburg, Germany).

Bromodeoxyuridine Incorporation

To test for DNA synthesis/repair by bromodeoxyuridine (BrdU) incorporation, the cells were resuspended in supplemented RPMI 1640 medium containing BrdU (20 μM) and E4031 (0 or 3 μM) irradiated with 0 or 5 Gy, post-incubated for 8 h at 37°C, washed, and fixed with 70% ethanol and consecutively treated with RNase A (0.1 mg/ml in PBS for 10 min at 37°C), pepsin (0.5 mg/ml in 0.05 N HCl for 10 min at 37°C), and 2 N HCl (for 10 min at room temperature). For immunolabelling, cells were incubated (30 min at room temperature) with a monoclonal mouse anti-BrdU antibody [1:67 dilution in phosphate buffered saline (PBS)/1% bovine serum albumin (BSA), Becton Dickinson, Pharmingen, Freiburg, Germany] and post-incubated (30 min at room temperature) with a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG antibody (1:100 in PBS/1% BSA, Dako, Hamburg, Germany). Thereafter, cells were stained (15 min at 4°C) with propidium iodide solution (25 μg/ml propidium iodide and 20 μg/ml RNAse A in PBS/1% BSA). BrdU- and propidium iodide-specific fluorescence were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany, 488 nm excitation wavelength) in fluorescence channels FL-1 (515–545 nm emission wavelength) and FL-3(> 670 nm emission wavelength), respectively. Data were analyzed with the FCS Express 3 software (De Novo Software, Los Angeles, CA, USA).

Cell Cycle Analysis in Flow Cytometry

K562 or primary CML cells were pre-incubated (30 min), irradiated (0 or 5 Gy), and incubated for further 24–72 h in supplemented RPMI 1640 medium additionally containing E4031 (0, 1, or 3 μM). For cell cycle analysis, cells were permeabilized and stained (30 min at room temperature) with Nicoletti propidium iodide solution (containing 0.1% Na-citrate, 0.1% Triton X-100, 10 μg/ml propidium iodide in PBS), and the DNA amount was analyzed by flow cytometry in fluorescence channel FL-3 (linear scale). In parallel, cells with degraded DNA were defined by the $subG_1$ population of the propidium iodide histogram recorded in fluorescence channel FL-2 (logarithmic scale, 564–606 nm emission wavelength).

Colony Formation Assay

To test for clonogenic survival, K562 cells were plated in six-well plates (n = 100 cells/well) in supplemented RPMI 1640 medium further containing E4031 (0 or 1 μM), irradiated (0, 2, 4, 6 Gy), and post-incubated for 2 weeks. Thereafter, colonies were defined as cluster of ≥ 50 cells and colony number N counted. Plating efficiency PE was defined by ratio between counted colonies and plated cells (PE = N/n). The survival fraction (SF) was calculated by normalizing in both arms (E4031 and vehicle control) separately the plating efficiency after irradiation (PE_{xGy}) to that of the corresponding unirradiated control (PE_{0Gy}) by the formula $SF = PE_{xGy}/PE_{0Gy}$.

γH2AX-Foci Formation

K562 cells were placed in cell culture medium containing E4031 (0 or 3 μM), irradiated (0 or 4 Gy), and post-incubated for 24 h. After several washing steps (PBS), cells were transferred on object slides by cytospin, fixed in 4% formaldehyde in PBS (3 x 5 min at room temperature), and cell membranes were solubilized with 0.1% Triton X-100 in PBS (3 x 5 min at room temperature), washed (PBS), quenched, blocked, incubated with anti-γH_2AX antibody (Upstate, Millipore, Billerica, MA, clone JBW301; 1:1,000 at room temperature for 1 h), as well as antibody binding visualized by fluorescence microscopy by the use of Alexa Fluor 488 Tyramide Super Boost Kit goat anti-mouse IgG (#B40912, Thermo Fisher Scientific, Schwerte, Germany) according to the protocols supplied by the manufacturer. After additional washing steps (PBS) cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma

Aldrich D9542, 25 μg/ml in PBS, 10 min at room temperature, dark conditions). Object slides were mounted in Fluorescence Mounting Medium (Dako, Carpinteria, CA) and z-stack fluorescence images (slice thickness = 0.5 μm, z-stacks = 11) were acquired using ApoTome.2 (Zeiss, Oberkochen, Germany). Nuclear γH2AX-foci were then counted in Maximum Intensity Projection images generated from the z-stacks.

Statistics

Data are given as means ± SE or individual values plus median, statistical significance was estimated by (Welch-corrected) two-tailed and one-sample t-test or for multiple pair comparisons by ANOVA. An error probability of $p \leq 0.05$ was considered statistically significant.

Safety Guidelines

All experimental protocols and research activities adhered to the standard biosecurity and institutional safety guidelines.

RESULTS

To test for radiation-stimulated hERG1 function in K562 cells, fast whole-cell currents of irradiated (0 or 5 Gy, 1–4 h post-irradiation) were recorded with the patch-clamp technique using K^+ and D-gluconate$^-$ as principle charge carriers in both pipette and bath solution. Since permeability of the plasma membrane for the large anion D-gluconate$^-$ is usually low, this configuration measures primarily K^+-selective (or nonselective cation) currents. hERG1 currents stand out due to their unusual gating characteristics: hERG1 channels activate slowly and inactivate rapidly during depolarizing voltage steps. Upon repolarization of the membrane voltage, release from inactivation occurs with faster kinetics than deactivation of the channels giving rise to deactivating tail currents (Vandenberg et al., 2012).

Here, we applied voltage pre-pulses delivered from −50 mV holding potential to voltages between −100 and +40 mV, followed by a hyperpolarizing voltage step to −100 mV (**Figure 1A**, insert). This hyperpolarization of the plasma membrane elicited deactivating tail inward currents (**Figure 1A**). Importantly, these tail currents were larger in pre-irradiated (5 Gy) than in control (0 Gy) cells (compare upper and lower line in **Figure 1A**). Moreover, bath administration of E4031 (1 μM) inhibited in a more or less reversible manner a fraction of the tail currents (**Figure 1A**, lower line) suggesting an involvement of hERG1 channels. **Figure 1B** shows the mean peak current densities at −100 mV voltage in dependence on the pre-pulse voltage of control (0 Gy, left) and irradiated (5 Gy, right) K562 cells recorded before (open circles) and during hERG1 inhibition (closed triangles). Irradiation significantly increased the E4031-sensitve fraction of deactivating tail current densities (compare black and red symbols in **Figure 1C**). Notably, ionizing radiation did not alter whole-cell capacities *c* as a measure membrane area ($c_{0Gy} = 11.5 \pm 0.5$ pF *vs.* $c_{5Gy} = 11.0 \pm 0.8$ pF, n = 9–16) suggesting that the increased hERG1 current did not just result from a radiation-induced increase in cell volume.

Radiogenic K^+ channel activity may modulate Ca^{2+} signaling. In the present study, we did not observe under control conditions gross differences in steady state fura-2 340/380 nm fluorescence ratio as a measure of $c[Ca^{2+}]_{free}$ between unirradiated and 5 Gy-irradiated K562 cells when recorded in the continuous presence of external Ca^{2+} (compare the initial parts of the graphs in **Figure 2A** between 0 Gy- and 5 Gy-irradiated cells). The low spatial/temporal resolution of our Ca^{2+} imaging set-up, however, was not suited to detect, e.g., local Ca^{2+} sparks or Ca^{2+} oscillations with frequencies faster than 0.1 Hz. Notably, E4031 evoked in unirradiated K562 cells a faint but significant increase in steady state $c[Ca^{2+}]_{free}$ (**Figure 2A**, upper line, closed symbols, and **Figure 2B**, 1st bar) while TEA did not alter steady state $c[Ca^{2+}]_{free}$ (**Figure 2A**, upper line, open symbols, and **Figure 2B**, 3rd bar). In irradiated cells, E4031 elicited a much stronger increase in $c[Ca^{2+}]_{free}$ than in unirradiated cells and TEA evoked a significant decrease of steady state $c[Ca^{2+}]_{free}$ (**Figure 2A**, lower line, **Figure 2B**, 2nd and 4th bars).

The latter observation might result from a Ca^{2+} leak of the plasma membrane that is stimulated in irradiated K562 cells by $K_v3.4$. As matter of fact, ionizing radiation has been demonstrated in our previous study (Palme et al., 2013) to accelerate Ca^{2+} re-entry in irradiated Ca^{2+}-depleted K562 cells in a $K_v3.4$-dependent manner: the $K_v3.4$ channel blocker TEA inhibited radiation-stimulated Ca^{2+} re-entry (Palme et al., 2013). To analyze the effect of hERG1 and combined hERG1 and $K_v3.4$ blockage on accelerate Ca^{2+} re-entry in irradiated Ca^{2+}-depleted K562 cells we applied in the present study a Ca^{2+} removal/re-addition protocol during fura-2 Ca^{2+} imaging (**Supplementary Figure 1A**). As a result, ionizing radiation (5 Gy) stimulated an increase of Ca^{2+} re-entry in Ca^{2+}-depleted cells from $s_{0Gy\text{-}control} = 0.4 \pm 0.03$ min^{-1} to $s_{5Gy\text{-}control} = 1.0 \pm 0.04$ min^{-1} (data are means ± SE, n = 167–267, and given as slope *s* of the increase in the fura-2 340/380 nm fluorescence ratio, **Supplementary Figure 1B**). E4031 when administered during Ca^{2+} re-entry augmented the basal ($s_{0Gy\text{-}E4031} = 0.9 \pm 0.05$ min^{-1}) and radiation-stimulated ($s_{5Gy\text{-}E4031}$ $s = 2.1 \pm 0.04$ min^{-1}) slope of Ca^{2+} re-entry. Co-administration of E4031 and the $K_v3.4$ inhibitor TEA during the Ca^{2+} re-entry, in sharp contrast, re-decreased slope of basal ($s_{0Gy\text{-}E4031+TEA} = 0.4 \pm 0.04$ min^{-1}) and radiation-induced ($s_{5Gy\text{-}E4031+TEA} = 0.7 \pm 0.07$ min^{-1}) Ca^{2+} re-entry to values observed in the control situations (data not shown) suggesting that $K_v3.4$ and hERG1 channels exert opposing effects on Ca^{2+} re-entry in Ca^{2+}-depleted cells. This is also illustrated by the calculated radiation-induced fraction of Ca^{2+} re-entry under control situation, in the presence of E4031, and in the presence of E4031 and TEA as shown in **Supplementary Figure 1C**. Combined, the Ca^{2+} removal/re-addition and the Ca^{2+} steady-state experiments of the present study suggest [also when taking into account our previously published data (Palme et al., 2013)] that hERG1 as well as $K_v3.4$ contribute to Ca^{2+} signaling in irradiated K562 cells. The opposing effects of hERG and $K_v3.4$ inhibition on Ca^{2+} re-entry and steady-state $c[Ca^{2+}]_{free}$ further suggest different functions of both channels in the Ca^{2+} signalosome of K562 cells.

Next, we analyzed by immunoblotting potential hERG-stimulated signaling pathways in K562 cells by analyzing the

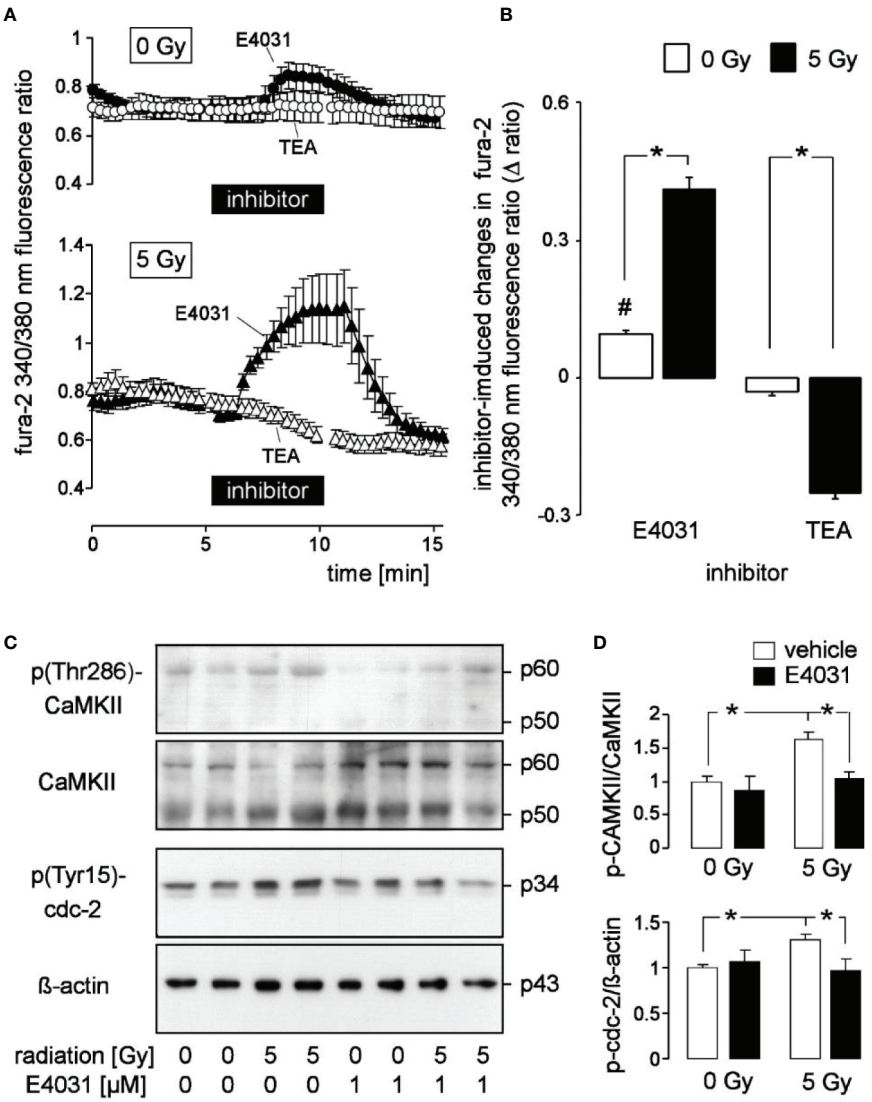


FIGURE 2 | Radiation augments in K562 cells the modulation of cytosolic free Ca^{2+} concentration ($c[Ca^{2+}]_{free}$) by the hERG1 inhibitor E4031 and the $K_v3.4$ blocker tetraethylammonium (TEA): effect of E4031 on Ca^{2+}-regulated signaling. **(A)** Time course of the mean (± SE, n = 7–10) ratio between the 340 nm- and the 380 nm-excited fura-2 fluorescence as a measure of $c[Ca^{2+}]_{free}$. Ratios were recorded 2–3 h post-irradiation with 0 Gy (upper line, n = 27) and 5 Gy-irradiated (lower line) K562 cells before, during, and after administration of E4031 (1 μM, closed triangles) or TEA (open circles). **(B)** Mean (± SE) change in fura-2 340/380 nm ratio (Δ ratio) elicited in 0 Gy (open bars) and 5 Gy (closed bars)-irradiated cells by E4031 (n = 90–111, left) and TEA (n = 57–59, right) application as recorded in **(A)**. **(C)** Immunoblots showing the abundance of phosphorylated (Thr286, first panel) and total CaMKII isoforms (second panel), phospho-(Tyr15)-cdc2 (third panel), or ß-actin (fourth panel) as loading control in total lysates of K562 cells cropped 2 h after irradiation with 0 and 5 Gy. Cells were irradiated and post-incubated in the absence or presence of the hERG-inhibitor E4031 (1 μM). **(D)** Mean (± SE, n = 3) normalized ratio between phospho-(Thr286)-CaMKII and total CaMKII protein abundance (top) and between phospho-(Tyr15)-cdc2 and ß-actin protein [densitometrically semi-quantified data from **(C)**]. # indicates different from 0, $p \leq 0.05$, one-sample t-test (**B**, 1st column); * indicates $p \leq 0.05$, two-tailed (Welch-corrected) t-test in **(B)** and ANOVA in **(D)**.

activities of the Ca^{2+} effector proteins CaMKIIs (Ca^{2+}/calmodulin-dependent protein kinase II isoforms) and their downstream target cdc2 (cyclin-dependent kinase 1, CDK1) (**Figures 2C, D**). Irradiation (5 Gy) stimulated activation of CaMKIIs and inactivation of their downstream target, cdc2 (cyclin-dependent kinase 1, CDK1) in K562. This was evident from phosphorylation at threonine 286 (CaMKIIs) and tyrosine 15 (cdc2), respectively (1st–4th lane in the immunoblots of **Figure 2C** and semi-quantified data shown as open bars in **Figure 2D**). Importantly, hERG1 inhibition by E4031 (1 μM) prevented radiation-induced CaMKIIs activation and cdc2 inactivation (**Figure 2C**, 5th–8th lane and **Figure 2D**, closed bars) suggesting that Ca^{2+} signals modulated by hERG1 were required for radiation-induced CaMKIIs activation and inhibition of cdc2.

Ca^{2+} signals *via* Ca^{2+} effector proteins such as the multifunctional CaMKI and -II isoforms reportedly control cell

cycle progression (Skelding et al., 2011). Therefore, cell cycle progression was analyzed in K562 cells by flow cytometry in dependence on irradiation and hERG1 inhibition. To this end, cells were irradiated with 0 or 5 Gy and post-incubated for 8 h in the presence of bromodeoxyuridine (BrdU) and E4031 (0 or 3 μM). Thereafter, cellular BrdU incorporation and DNA content were visualized by immunofluorescence- and propidium iodide staining, respectively, in flow cytometry experiments (**Figure 3A**). As a result, radiation decreased both G_1 populations (without and with incorporated BrdU) and increased the BrdU-incorporating S population, increased in tendency BrdU-negative G_2 population, but left BrdU-positive G_2 population unchanged (compare open and closed bars in **Figure 3B**). This might be explained by a slowed-down S progression paired with a beginning accumulation of the irradiated cells at the G_2/M border of cell cycle, or a combination of both. Remarkably, hERG1 inhibition did not modify the radiation-induced decrease of both G1 populations. Instead, E4031 decreased the radiation induced accumulation of BrdU incorporating S phase cells and showed a trend to increase both G_2 populations (**Figure 3C**), suggestive of a hERG1 function in the radiation-induced S phase arrest during the first 8 h after irradiation.

Next, we studied in K562 cells by flow cytometry using propidium iodine single staining (**Figure 4A**) a potential hERG1 function in cell cycle progression at later time points (24–72 h) after irradiation (0 or 5 Gy). The dynamics of the irradiated cells residing in G_1, S, or G_2 phases of cell cycle (**Figure 4B**, open circles) suggest that the proposed initial delay in S phase progression seen at 8 h (see **Figure 3B**) and at 24 h after irradiation transitioned into a G_2/M arrest (**Figure 4B**, open circles). Later on, the radiation induced decrease of the G_1 population and the increases in S and G_2 population regressed again, reaching (almost) basal levels 72 h after irradiation (**Figure 4B**, open circles). hERG1 inhibition with E4031 (3 μM) blunted in the irradiated K562 cells the S phase arrest at 24 h, and accelerated the recovery of G_1 population and release from G_2/M arrest between 24 and 72 h after irradiation (**Figure 4B**, closed triangles). In unirradiated K562 cells, E4031 (3 μM for 48 h) slightly increased G_1 and decreased S population (**Figure 4C**, 1st and 2nd bars) with lower (G_1) or similar effect size as compared to its action in irradiated cells (**Figure 4C**, 3rd and 4th bars). Combined, the data of **Figures 3** and **4** suggest an involvement of hERG1 in the regulation of cell cycle progression. In particular, in irradiated K562 cells, hERG1 inhibition blunted S and G_2/M cell cycle arrest.

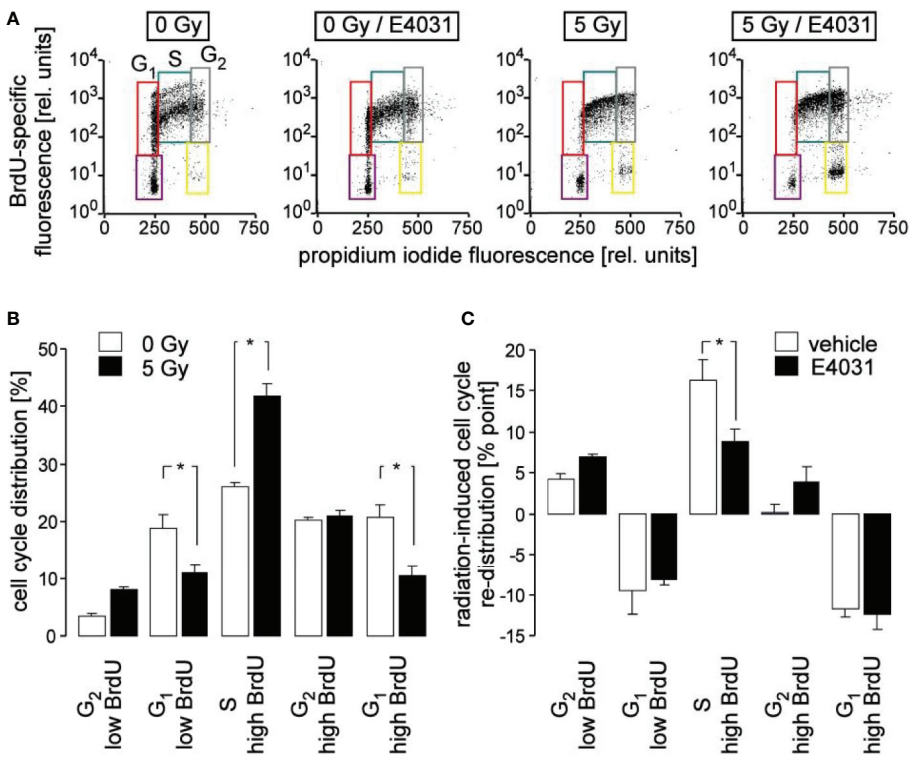


FIGURE 3 | Inhibition of hERG channels stimulates release from S phase arrest. **(A)** Flow cytometry dot plots showing DNA incorporation of the base analogon bromodeoxyuridine (BrdU) and DNA amount by propidium iodide staining during 8 h of incubation following irradiation with 0 Gy (1st and 2nd plot) and 5 Gy (3rd and 4th plot). Irradiation (0 and 5 Gy) and post-incubation (8 h) were carried out either in the absence (1st and 3rd plot) or presence (2nd and 4th plot) of the hERG channel inhibitor E4031 (3 μM). Cells which did not accumulate BrdU and which resided in G_1 and G_2 phase of cell cycle are outlined in violet and yellow, respectively. BrdU-positive cells which resided in G_1, S and G_2 phase of cell cycle are outlined in red, turquoise, and gray, respectively. **(B, C)** Mean (± SE, n = 8–9) normalized percentage **(B)** and radiation (5 Gy)-induced cell cycle re-distribution (mean percent points ± SE, n = 8–9) of K562 cells **(C)** incubated in the absence (open bars, vehicle) and presence of E4031 (closed bars) residing in the G_2 low BrdU, G_1 low BrdU, S, G_2 high BrdU, or G_1 high BrdU population as defined in **(A)**. * indicates $p \leq 0.05$, ANOVA.

Entry in mitosis with unfixed DNA double strand breaks may result in chromosome aberration and eventually in mitotic catastrophe. To avoid such immature mitosis entry, irradiated cells arrest their cell cycle to gain time for DNA double strand repair. Upon hERG1 inhibition, the observed accelerated release from S and G_2/M cell cycle arrest might, therefore, impair the survival of irradiated K562 cells. To test for such a scenario, the population of K562 cells with degraded DNA ($subG_1$ population) was determined in dependence on hERG1 inhibition (0 or 1 μM E4031) by flow cytometry (propidium iodide Nicoletti staining) 24 h after irradiation with 0 or 5 Gy (**Figure 5A**). As a result, E4031 almost doubled the percentage of dead irradiated cells while having no effect on unirradiated K562 cells (**Figure 5B**). Furthermore, clonogenic survival of irradiated (0, 2, 4, or 6 Gy) K562 cells was determined again in dependence on hERG1 channel inhibition in pre-plating colony formation assay. For this assay, K562 cells were irradiated (0–6 Gy), and thereafter incubated in the absence or presence of E4031 (1 μM) until formation of colonies. For calculation of survival fractions, plating efficiencies (i.e., number of colonies divided by number of plated cells) were normalized to that of the respective 0 Gy control value separately in the E4031 and vehicle treatment arm. The survival fractions were plotted against the radiation dose demonstrating that E4031 decreased survival fractions and, hence, radiosensitized the K562 cells (**Figure 5C**). To test, whether this E4031-mediated radiosensitization is associated with impaired DNA repair, γH2AX foci [phospho-(139S)-H2A histone family member X] were determined as a surrogate marker of residual (= un-repaired) DNA double strand breaks (DSBs) by immunofluorescence microscopy 24 h after irradiation with 0 or 4 Gy (**Figure 5D**). E4031 (3 μM) did not increase the number of residual γH2AX foci (**Figures 5E, F**) suggesting that hERG signaling is not involved in DNA DSB repair. Combined, the data indicate that hERG1 channels contribute to the stress response and clonogenic survival of irradiated K562 cells but do not promote DNA repair.

Mitochondria have been identified to contribute to radiation-induced cell damage by formation of superoxide anion radicals

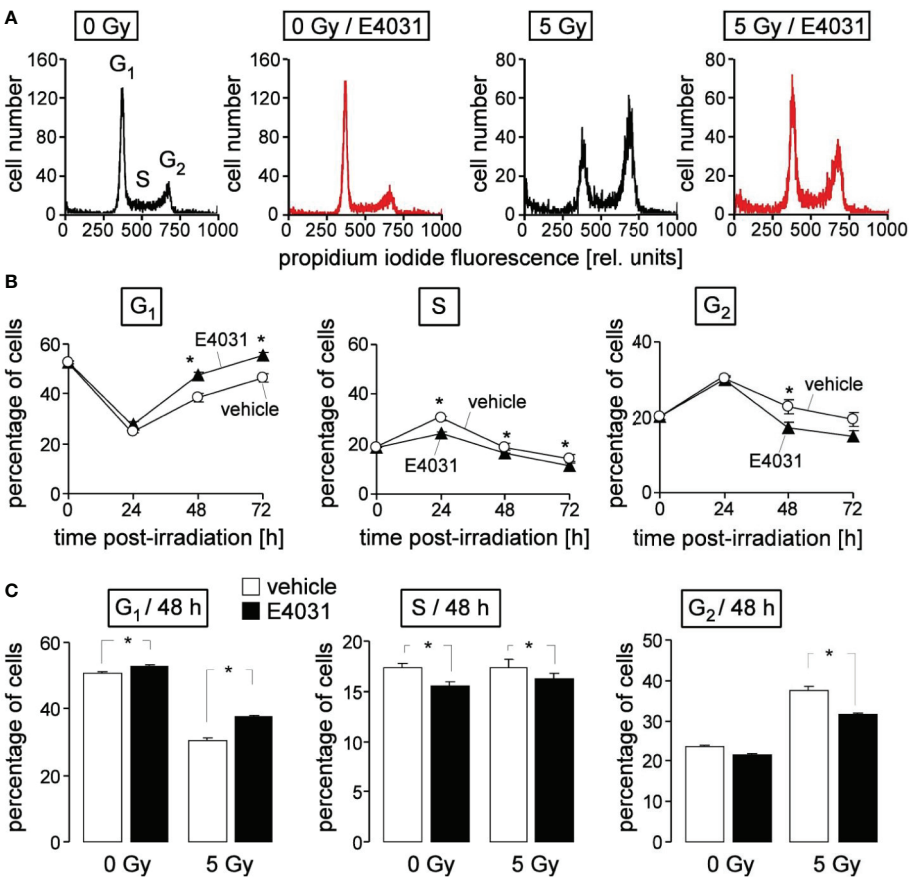


FIGURE 4 | hERG channels delay recovery from radiation-induced release from S and G_2/M arrest in K562 cells. **(A)** Flow cytometry histograms depicting the propidium iodide fluorescence (Nicoletti staining) of K562 cells irradiated (0 or 5 Gy) and post-incubated (48 h) with vehicle (black) or hERG inhibitor E4031 (3 μM). **(B)** Time course of radiation (5Gy)-induced changes in cell cycle progression in the absence (vehicle, open circles) and presence of E4031 (3 μM, closed triangles). Shown are mean (± SE, n = 21) percentages of cells residing in G_1 (left), S (middle), or G_2 (right) phase of cell cycle 0, 24, 48, and 72 h after irradiation. **(C)** Mean percentage (± SE, n = 27) of irradiated (0 or 5 Gy) K562 cells residing 48 h after irradiation in G_1 (left), S (middle), or G_2 (right) phase of cell cycle. Cells were pre- and post-incubated with vehicle (open bars) or E4031 (3 μM, closed bars). * indicates $p \leq 0.05$, two-tailed t-test in **(B)** and ANOVA in **(C)**.

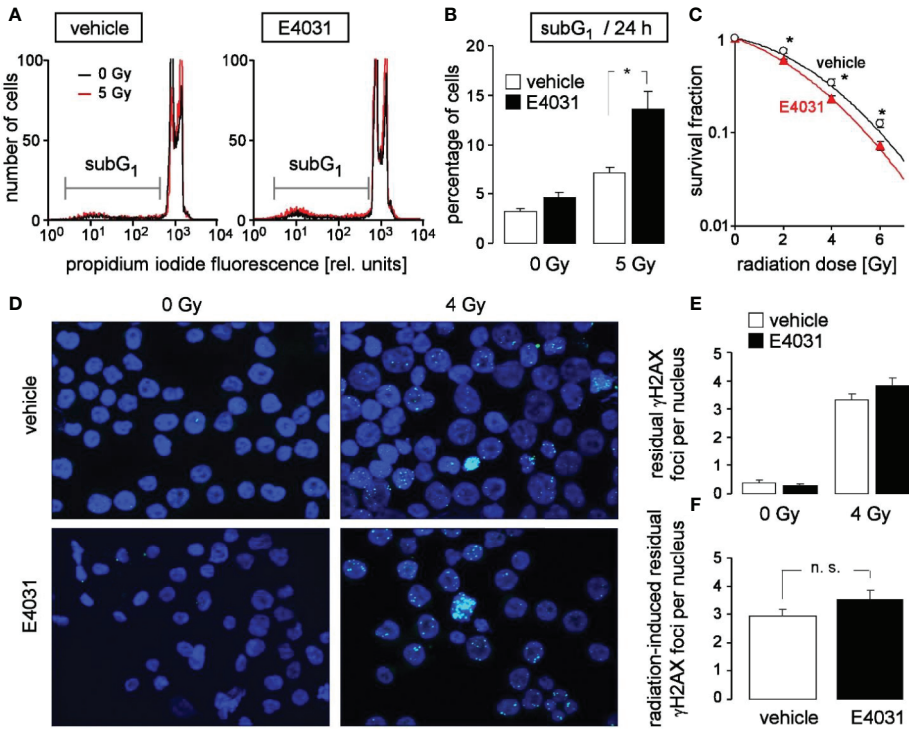


FIGURE 5 | hERG channels confer radioresistance to K562 cells without promoting DNA repair. **(A)** Flow cytometry histograms highlighting the dead population of irradiated (0 Gy in black and 5 Gy in red) K562 cells with low (subG1) propidium iodide fluorescence (Nicoletti staining). K562 cells were irradiated (0 or 5 Gy) and post-incubated (24 h) in the absence (vehicle, left) or presence of hERG inhibitor E4031 (1 μM, right). **(B)** Mean (± SE, n = 9) percentage of dead ($subG_1$) K562 cells 24 h after irradiation with 0 (left) or 5 Gy (right) and co-incubation with vehicle alone (open bars) or E4031 (1 μM, closed bars). **(C)** Mean (± SE, n = 12–24) survival fraction of irradiated (0, 2, 4, 6 Gy) K562 cells as determined by pre-plating colony formation assay. Cells were irradiated and post-incubated in the absence (vehicle, black) or presence of E4031 (1 μM, red). **(D)** Fluorescence micrographs of 0 Gy- (left) and 4 Gy-irradiated (closed symbols) K562 cells immunostained against γH2AX (green) and counterstained against DNA with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Cells were irradiated and 24 h post-incubated in the absence or presence of E4031 (3 μM) before analysis. **(E, F)** Mean (± SE, n = 193–273) number of residual nuclear γH2AX foci 24 h after irradiation with 0 (left) or 4 Gy (right) and co-treatment with 0 (open bars) or 3 μM E4031 (closed bars) **(E, F)** of the radiation-induced residual nuclear γH2AX foci 24 h after co-treatment with 0 (open bars) or 3 μM E4031 (closed bars).* and n.s. indicate $p \leq 0.05$ and not significant different, respectively, ANOVA in **(B)** and two-tailed (Welch-corrected) t-test in **(C, F)**.

[for review see (Eckert et al., 2019)]. To define a potential role of hERG1 and $K_v3.4$ channels herein modulation of inner mitochondrial membrane potential ($\Delta\Psi_m$) and mitochondrial formation of superoxide anion radicals were determined by flow cytometry 24 h after irradiation with 0 or 8 Gy (**Supplementary Figures 2** and **3**). Irradiation and 24 h post-incubation were carried out in the absence or presence of E4031 or TEA, or both inhibitors. As a result, applying the combination of both drugs pronounced hyperpolarization of $\Delta\Psi_m$ and formation of superoxide anions in the irradiated cells. For the latter an additive effect of E4031 and TEA was observed. Together, these data confirmed the contribution of both channels in the stress response of irradiated K562 cells.

Finally, primary chronic myeloid leukemia (CML) cells were tested for hERG1 function in the irradiation-induced stress response. Fast whole cell patch clamp recording revealed E4031-sensitive, time-dependently deactivating inward tail currents at −80 mV clamp voltage that exhibited the hERG1-typical dependence on holding potential (**Figure 6A**). The tail currents were larger in irradiated (5 Gy, 2–4 h after irradiation) as compared to control (0 Gy) primary CML cells. This was evident from the peak current densities shown for 0 Gy- and 5 Gy-irradiated cells recorded with -40 mV and +50 mV holding potential (**Figure 6B**) and the thereof calculated holding potential-dependent fraction of peak current densities (**Figure 6C**).

To define the functional significance of radiation-stimulated hERG1-like channel activity, hERG1-dependent cell cycle progression of irradiated (0 or 5 Gy) primary CML cells was assessed by flow cytometry with propidium iodide Nicoletti staining of the DNA 24 h after irradiation (**Figure 6D**). Radiation induced in these cells a decrease of G_1 and an increase in G_2 population, suggestive of a G_2/M arrest (**Figure 6E**, open bars). Importantly, the hERG inhibitor E4031 (3 μM) which was administered during irradiation and the following 24 h post-incubation time blunted the G_2/M cell cycle arrest (**Figure 6E**, closed bars). This suggests that radiation-induced hERG1 activity contributes to the cellular stress response in primary CML cells similar to its function observed in K562 cells (compare **Figure 4**).

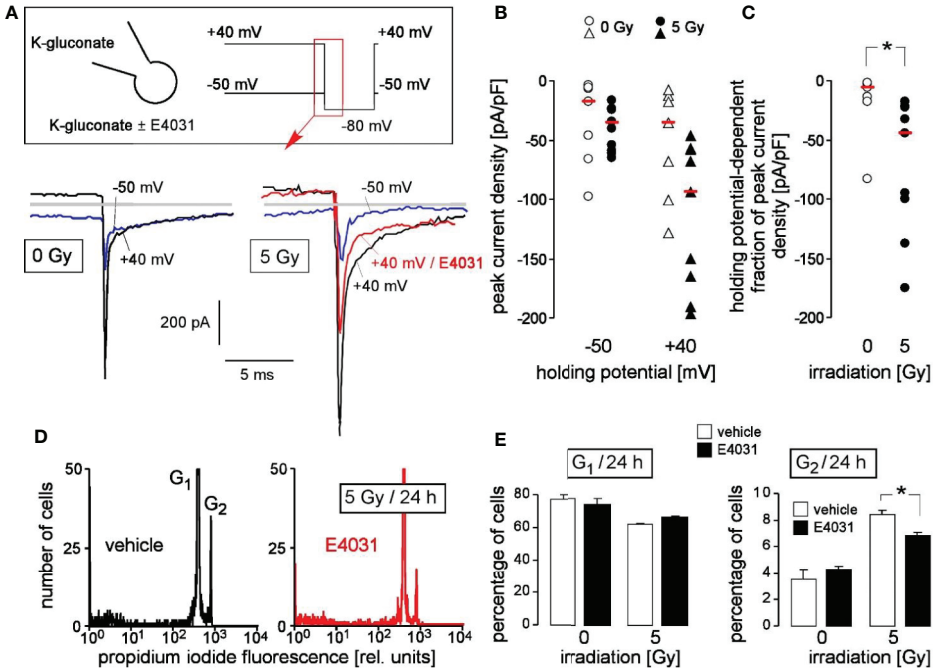


FIGURE 6 | Primary chronic myeloid leukemia (primary CML) cells express radiation-induced hERG currents: involvement in G_2/M cell cycle arrest. **(A)** Whole-cell deactivating tail current tracings from a control (0 Gy, left) and an irradiated primary CML cell (5 Gy, right). Records were obtained with K-D-gluconate pipette and Na-D-gluconate bath solution (insert). Square pulses to –80 mV (insert) were either delivered from a holding potential of +40 mV (black tracings) or –50 mV (blue tracings) as indicated. The current tracing in red color was recorded during administration of E4031 (3 μM). Gray lines indicate zero current. **(B, C)** Peak current densities of the deactivating tail current after stepping the clamp voltage to –80 mV either from a holding potential of –50 or +40 mV **(B)** and holding potential-dependent peak current density fractions of the deactivating tail current **(C)** of control (0 Gy, open symbols) or 5 Gy-irradiated primary (closed symbols) CML cells. Symbols and red lines indicate individual values (n = 7–9) and medians, respectively. **(D)** Flow cytometry histograms showing the propidium iodide fluorescence (Nicoletti staining) of primary CML cells that were irradiated (5 Gy) and post-incubated (24 h) with vehicle (left) or hERG inhibitor E4031 (3 μM, right). **(E)** Mean (± SE, n = 3–4) percentage of irradiated (0 or 5 Gy) primary CML cells residing 24 h after irradiation in G_1 (left) or G_2 (right) phase of cell cycle (right). Cells were pre- and post-incubated with vehicle (open bars) or E4031 (3 μM, closed bars). * indicates $p \leq 0.05$, two-tailed (Welch-corrected) t-test in **(C)** and ANOVA in **(E)**.

DISCUSSION

The present study disclosed in the K562 cell line and in primary leukemic cells a function of hERG1 channels for cell cycle control in irradiated CML cells. In K562, radiogenic hERG1 activity contributed to clonogenic survival of the irradiated cells. Activation of K^+ channels by ionizing radiation has been reported in different tumor entities. In particular, radiation-stimulated activation of Ca^{2+}-dependent high conductance BK ($K_{Ca}1.1$, KCNMA1) (Steinle et al., 2011) and intermediate conductance IK ($K_{Ca}3.1$, SK4, KCNN4) K^+ channels (Stegen et al., 2016) occurs in glioblastoma cells. Radiation stimulated activity of the latter channels has been observed also in human T cell leukemia cells (Klumpp et al., 2016; Voos et al., 2018), in human lung adenocarcinoma (Huber et al., 2012; Roth et al., 2015), and in murine breast cancer cells (Mohr et al., 2019). $K_v3.4$ (KCNC4) voltage-gated K^+ channels (Palme et al., 2013) are activated by ionizing radiation together with hERG1 channels (present study) in K562 cells.

Radiation-induced activation mechanisms may comprise membrane lipid peroxidation and subsequent activation of tyrosine kinases such as src (Dittmann et al., 2009) and Pyk2 kinase (Proudfoot et al., 2018). The latter kinase has been demonstrated to directly modulate BK channels (Stegen et al., 2016). In addition, ionizing radiation has been reported to stabilize hypoxia-inducible factor-1α (HIF1α) which, in turn, upregulates auto-/paracrine chemokine signaling *via* the stromal-cell-derived factor-1 (SDF1, CXCL12)/CXCR4 axis (Edalat et al., 2016). CXCR4 reportedly signals through formation of inositol triphosphate and releases Ca^{2+} from intracellular stores (Hadad et al., 2013), which can be assumed to activate Ca^{2+}-dependent K^+ channels such as IK or BK [for review see (Eckert et al., 2018)]. Beyond that, data in Jurkat T cell leukemia cells suggest that radiogenic activity of IK channels is under the tight control of anti-apoptotic proteins such as Bcl-2 (Klumpp et al., 2016).

hERG channels reportedly contribute to outside-in signaling in complexes with β1 integrin (see *Introduction*) and CXCR4 in the plasma membrane. In addition to radiogenic CXCR4 activation (Pillozzi et al., 2011), ionizing radiation may upregulate integrin β1 signaling by increasing expression and clustering (Voos et al., 2018). One might speculate that radiogenic upregulation of these signaling pathways might underlie the increased hERG1 function in irradiated K562 cells observed in the present study. Combined, this hints to a highly

complex regulation mechanisms of Ca^{2+}-dependent and voltage-gated K^+ channel activity in irradiated cells.

Radiogenic K^+ channel activity has been suggested to drive Na^+/glucose cotransport across the plasma membrane contributing to glucose fueling of the irradiated cell (Huber et al., 2012). This probably provides extra energy and carbohydrates, the latter required for chromatin remodeling by histone acetylation resulting in DNA decondensation as a prerequisite of DNA damage repair (Dittmann et al., 2013). Moreover, radiogenic K^+ channel activity reportedly is needed for the repair of radiation-induced DNA double strand breaks (Stegen et al., 2015; Klumpp et al., 2018). Furthermore, radiogenic K^+ channel activity has been shown to stimulate hypermigration of glioblastoma cells (Steinle et al., 2011; Edalat et al., 2016).

Radiogenic K^+ channel-mediated electrosignaling reportedly acts both downstream and upstream of Ca^{2+} signaling (Stegen et al., 2016). In theory, Ca^{2+} entry may result in local depolarization of the plasma membrane as well as in local rise in $c[Ca^{2+}]_{free}$ beneath the plasma membrane which trigger activation of physically associated voltage-gated K^+ channels and Ca^{2+}-dependent K^+ channels, respectively. K^+ channel activity, in turn, maintains the inwardly directed electrochemical driving force for Ca^{2+} entry and/or modulates activity of voltage-regulated Ca^{2+} entry pathway. For instance, in Jurkat T cell leukemia and K562 CML cells, TRPM2 (member 2 of the melastatin family of transient receptor potential) (Klumpp et al., 2016) and TRPV5/6-like (member 5/6 of the vanilloid family of TRP) Ca^{2+}-permeable nonselective cation channels (Heise et al., 2010) have been proposed to generate radiogenic Ca^{2+} signals in concert with IK (Klumpp et al., 2016) and $K_v3.4$ (Palme et al., 2013) K^+ channels, respectively. The present study provides evidence that also radiogenic hERG1 activity contributes to those Ca^{2+} signals in K562 cells.

Notably, $K_v3.4$ blockage reportedly delays Ca^{2+} re-entry into Ca^{2+}-depleted K562 cells (Palme et al., 2013) and decreased in the present study in irradiated cells steady state $c[Ca^{2+}]_{free}$ while hERG1 boosted Ca^{2+} re-entry into Ca^{2+}-depleted K562 cells and increased $c[Ca^{2+}]_{free}$. At the first glance, these opposing effects seem to be counterintuitive since inhibition of both K^+ channel types is assumed to depolarize the membrane potential of the plasma membrane. One (merely speculative) scenario that might results in opposing K^+ channel effects in K562 cells assumes that $K_v3.4$ channels might be in complex with, e.g., store-operated Ca^{2+} channels and maintain the inwardly directed electrochemical driving force for Ca^{2+} entry by counteracting the Ca^{2+} influx-elicited strong local depolarization of the plasma membrane. hERG1 channels, in contrast might be in complex with further Ca^{2+} entry pathways where they inhibit these pathways either in a non-conductive (i.e., by direct protein-protein interaction) or conductive manner. For the latter, one has to postulate that hERG1 functionally interact with voltage-dependent Ca^{2+} entry pathways that exhibit increasing activity/conductivity with increasing depolarization of the membrane potential.

Likewise, in glioblastoma cells, TRPM8 channels collaborate with Ca^{2+}-dependent IK and BK channels (Klumpp et al., 2017) to generate radiogenic Ca^{2+} signals. Similarly to the proposed antagonistic action of $K_v3.4$ and hERG1 in K562 cells, IK channel inhibition either decreases steady state $c[Ca^{2+}]_{free}$ or induces oscillation in $c[Ca^{2+}]_{free}$ in glioblastoma cells while BK channel blockage results in an rapid increase in steady state $c[Ca^{2+}]_{free}$ (Stegen et al., 2016). Together, these observations strongly suggest that the radiogenic activities of several K^+ and Ca^{2+}-permeable channel types collaborate to fine tune diverse Ca^{2+} signals in the irradiated cells. Since $K_v3.4$ and hERG1 operate during different voltage alterations ($K_v3.4$ upon depolarization and hERG1 upon repolarization of the membrane potential) with different kinetics it is hardly surprising that they exert different functions in the Ca^{2+} signalosome.

CaMKII kinases have been identified as downstream targets of radiogenic K^+ and TRP channel activity [for review see (Stegen et al., 2016)]. Ca^{2+} signals are versatile comprising Ca^{2+} transients, long-lasting Ca^{2+} elevations, oscillations differing in amplitude and frequency, etc. that are formed in a time-spatial dependent manner and that have different meanings for the cells. CaMKII isoforms reportedly are encoded by four genes and expressed in several splice variants that form homo- or hetero-dodecameric CaMKII holoenzymes some of them with nuclear localization signal which exhibit different substrate specificity and an intriguingly complex form of regulation by Ca^{2+}/CaM. The latter involves activating and inhibiting autophosphorylation and modulation of CaM binding. Upon stimulatory autophosphorylation at threonine 286 following Ca^{2+}/CaM binding, CaMKIIs stay active in the absence of Ca^{2+}/CaM, thus, transducing short-term electro- and Ca^{2+} signals in long-lasting biochemical signaling [for review see (Swulius and Waxham, 2008; Coultrap and Bayer, 2012)].

Although resulting in a decrease and increase of $c[Ca^{2+}]_{free}$, respectively, inhibition of either hERG1 or $K_v3.4$ impaired radiogenic activation of CaMKIIs in K562 cells [(Palme et al., 2013) and present study]. These on the first view conflicting data might reflect a highly complex regulation of the CaMKIIs by Ca^{2+} that require both, $K_v3.4$ and hERG1 channels. Nucleus-translocated CaMKII isoforms have shown previously and in the present study to regulate cell cycle in irradiated K562 CML [(Heise et al., 2010) and present study], Jurkat T cell leukemia (Klumpp et al., 2016), and glioblastoma cells (Klumpp et al., 2017). CaMKII-mediated inhibitory phosphorylation of cdc25 phosphatases [cdc25B in K562 CML (Palme et al., 2013) and Jurkat T cell leukemia (Klumpp et al., 2016), and probably cdc25C in glioblastoma cells (Klumpp et al., 2017)] prevents the activating dephosphorylation of the cdc25 target p-(Tyr15)-cdc2 as also observed in the present study. Cdc2 together with cyclin B forms the mitosis-promoting factor, and the inhibitory phosphorylation results in G_2/M arrest of the cell cycle (Palme et al., 2013; Klumpp et al., 2016; Klumpp et al., 2017).

Importantly, hERG1 as well as $K_v3.4$ and IK seem to be critically required not only for CaMKIIs activation but also for cell cycle arrest in irradiated CML [(Palme et al., 2013) and present study), T cell leukemia (Klumpp et al., 2016), and glioblastoma cells (Stegen et al., 2015)], since pharmaceutical or molecular biological targeting of these channels overrides cell cycle arrest resulting in premature (i.e., with unfixed DNA double strand breaks) entry in mitosis and cell death. In addition to cell cycle control, radiogenic hERG1 and

$K_v3.4$ activities apparently are required to blunt mitochondrial hyperpolarization and superoxide anion formation in irradiated K562 cells (see **Supplementary Figures 2** and **3**). Since mitochondrial Ca^{2+} overload may trigger radiogenic mitochondrial superoxide anion formation [for review see (Eckert et al., 2019)] one might speculate that radiogenic hERG1 and $K_v3.4$ activities interfere with Ca^{2+} homeostasis during DNA damage response beyond regulating the Ca^{2+}/CaM/CaMKII pathway.

Combined, these observations suggest K^+ channels such as hERG1 and IK that are upregulated in several tumor entities as a very attractive target for anti-cancer therapy. Preclinical mouse studies have proven the efficacy of an anti-hERG1 therapy strategy (Pillozzi et al., 2011; Crociani et al., 2013; Crociani et al., 2014; Pillozzi et al., 2018). However, since hERG1 contributes to the repolarization of the heart action potential, so-called "torsadogenic" hERG blockers may delay cardiac repolarization and, therefore, substantially increases the risk of Torsade de Pointes and sudden cardiac death. On the other hand, several drugs in clinical use (among those are antihistamines, fluoroquinolone antibiotics, antipsychotics, antiepileptics) exert off-target inhibitory effects on hERG1 partially with submicromolar IC_{50}s but are "non-torsadogenic." One might assume that clinically achieved steady state free plasma concentrations of these "non-torsadogenic" hERG1 inhibitors remain far below its IC_{50} concentration for hERG1 (Lehmann et al., 2018). Another explanation for the "non-torsadogenicity" of these drugs is that hERG1 inhibition alone is not sufficient to induce fatal arrhythmia, and the "torsadogenic" action of a certain drug depends on the full spectrum of ion channels that is modulated by the drug [for commentary see (Arcangeli and Becchetti, 2017)]. Nevertheless, "non-torsadogenic" hERG1 inhibitors might impact tumor biology. Along those lines, a retrospective multivariate analysis of glioblastoma specimens defined hERG1 protein abundance in the tumor as an independent prognostic parameter for shorter patient survival. Notably, patients with highly hERG1-expressing glioblastoma but not those with low expression showed in univariate analysis a benefit in terms of overall survival from "non-torsadogenic" hERG1-inhibiting drugs (Pointer et al., 2017).

Moreover, plasmalemmal hERG1/beta1-integrin complexes seem to be specific for tumor cells (Pillozzi and Arcangeli, 2010) which—at least in theory—increases the possibility to develop tumor-specific inhibitors that target hERG1/beta1-integrin complexes. Finally, the truncated, N-deleted splice variant hERG1b is reportedly up-regulated during the S phase of cell cycle, while the full-length hERG1a protein increases its expression on the plasma membrane during the G1 phase (Crociani et al., 2003). Consequently, proliferating tumor cells should exhibit higher hERG1b/1a expression ratios than terminally differentiated cardiomyocytes (Larsen et al., 2008) as has indeed been reported for some tumors (Erdem et al., 2015). Inhibitor selectivity for variant 1b over 1a (Gasparoli et al., 2015) might be a further strategy to develop tumor-specific hERG1 blockers. Taken together, circumventing cardiotoxic effects of pharmacological hERG1 blockade seems to become feasible in the near future which would classify hERG1 as druggable target in anti-cancer therapy.

In conclusion, irradiated CML cells utilize hERG1-modulated Ca^{2+}-signals during DNA damage response. These Ca^{2+} signals contribute *via* the CaMKIIs/cdc2 pathway to cell cycle arrest. Inhibition of hERG1 by the class III antiarrhythmic agent E4031 overrides cell cycle arrest and impairs clonogenic survival of the cells suggesting hERG1 as potential therapy target.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethik-Kommission an der Medizinischen Fakultät der Eberhard-Karls-Universität und am Universitätsklinikum Tübingen. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Research design: SH. Conducted experiments: DP, MM, KG, LK. Data analysis: DP, MM, KG, LK, SH. Contributed to discussions: HS, DZ. Wrote or contributed to the writing of the manuscript: HS, DZ, SH. Edited the manuscript: SH. Approved the content and submission of the paper: all authors.

FUNDING

This project was supported by a grant of the German Cancer Aid (70112872/70113144). DP was supported by the DFG International Graduate School 1302,). LK was supported by the ICEPHA program of the University of Tübingen and the Robert-Bosch-Gesellschaft für Medizinische Forschung, Stuttgart, Germany.

ACKNOWLEDGMENTS

We thank Heidrun Faltin and Ilka Müller for excellent technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00489/full#supplementary-material

REFERENCES

Arcangeli, A. (2005). Expression and role of hERG channels in cancer cells. *Novartis Found Symp.* 266, 225–32; discussion 232-4. doi: 10.1002/047002142X.ch17

Arcangeli, A., and Becchetti, A. (2017). hERG Channels: From Antitargets to Novel Targets for Cancer Therapy. *Clin. Cancer Res.* 23 (1), 3–5. doi: 10.1158/1078-0432.Ccr-16-2322

Barry, P. H., and Lynch, J. W. (1991). Liquid junction potentials and small cell effects in patch-clamp analysis. *J. Membr. Biol.* 121 (2), 101–117. doi: 10.1007/BF01870526

Becchetti, A., Petroni, G., and Arcangeli, A. (2019). Ion Channel Conformations Regulate Integrin-Dependent Signaling. *Trends Cell Biol.* 29 (4), 298–307. doi: 10.1016/j.tcb.2018.12.005

Choi, K. H., Song, C., Shin, D., and Park, S. (2011). hERG channel blockade by externally applied quaternary ammonium derivatives. *Biochim. Biophys. Acta* 1808 (6), 1560–1566. doi: 10.1016/j.bbamem.2011.02.008

Coultrap, S. J., and Bayer, K. U. (2012). CaMKII regulation in information processing and storage. *Trends Neurosci.* 35 (10), 607–618. doi: 10.1016/j.tins.2012.05.003

Craddock, C. F. (2018). We do still transplant CML, don't we? *Hematol. Am. Soc. Hematol. Educ. Program* 2018 (1), 177–184. doi: 10.1182/asheducation-2018.1.177

Crociani, O., Guasti, L., Balzi, M., Becchetti, A., Wanke, E., Olivotto, M., et al. (2003). Cell cycle-dependent expression of HERG1 and HERG1B isoforms in tumor cells. *J. Biol. Chem.* 278 (5), 2947–2955. doi: 10.1074/jbc.M210789200

Crociani, O., Zanieri, F., Pillozzi, S., Lastraioli, E., Stefanini, M., Fiore, A., et al. (2013). hERG1 channels modulate integrin signaling to trigger angiogenesis and tumor progression in colorectal cancer. *Sci. Rep.* 3, 3308. doi: 10.1038/srep03308

Crociani, O., Lastraioli, E., Boni, L., Pillozzi, S., Romoli, M. R., D'Amico, M., et al. (2014). hERG1 Channels Regulate VEGF-A Secretion in Human Gastric Cancer: Clinicopathological Correlations and Therapeutical Implications. *Clin. Cancer Res.* 20, 1502–1512. doi: 10.1158/1078-0432.CCR-13-2633

Dittmann, K., Mayer, C., Kehlbach, R., Rothmund, M. C., and Peter Rodemann, H. (2009). Radiation-induced lipid peroxidation activates src kinase and triggers nuclear EGFR transport. *Radiother. Oncol.* 92 (3), 379–382. doi: 10.1016/j.radonc.2009.06.003

Dittmann, K., Mayer, C., Rodemann, H. P., and Huber, S. M. (2013). EGFR cooperates with glucose transporter SGLT1 to enable chromatin remodeling in response to ionizing radiation. *Radiother. Oncol.* 107 (2), 247–251. doi: 10.1016/j.radonc.2013.03.016

Eckert, F., Schilbach, K., Klumpp, L., Bardoscia, L., Sezgin, E. C., Schwab, M., et al. (2018). Potential Role of CXCR4 Targeting in the Context of Radiotherapy and Immunotherapy of Cancer. *Front. Immunol.* 9, 3018. doi: 10.3389/fimmu.2018.03018

Eckert, F., Zwirner, K., Boeke, S., Thorwarth, D., Zips, D., and Huber, S. M. (2019). Rationale for Combining Radiotherapy and Immune Checkpoint Inhibition for Patients With Hypoxic Tumors. *Front. Immunol.* 10, 407. doi: 10.3389/fimmu.2019.00407

Edalat, L., Stegen, B., Klumpp, L., Haehl, E., Schilbach, K., Lukowski, R., et al. (2016). BK K+ channel blockade inhibits radiation-induced migration/brain infiltration of glioblastoma cells. *Oncotarget* 7 (12), 14259–14278. doi: 10.18632/oncotarget.7423

Erdem, M., Tekiner, T. A., Fejzullahu, A., Akan, G., Anak, S., Saribeyoglu, E. T., et al. (2015). herg1b expression as a potential specific marker in pediatric acute myeloid leukemia patients with HERG 897K/K genotype. *Pediatr. Hematol. Oncol.* 32 (3), 182–192. doi: 10.3109/08880018.2014.949941

Gasparoli, L., D'Amico, M., Masselli, M., Pillozzi, S., Caves, R., Khuwaileh, R., et al. (2015). New pyrimido-indole compound CD-160130 preferentially inhibits the KV11.1B isoform and produces antileukemic effects without cardiotoxicity. *Mol. Pharmacol.* 87 (2), 183–196. doi: 10.1124/mol.114.094920

Hadad, I., Veithen, A., Springael, J. Y., Sotiropoulou, P. A., Mendes Da Costa, A., Miot, F., et al. (2013). Stroma cell-derived factor-1alpha signaling enhances calcium transients and beating frequency in rat neonatal cardiomyocytes. *PloS One* 8 (2), e56007. doi: 10.1371/journal.pone.0056007

Heise, N., Palme, D., Misovic, M., Koka, S., Rudner, J., Lang, F., et al. (2010). Non-selective cation channel-mediated Ca2+-entry and activation of Ca2+/calmodulin-dependent kinase II contribute to G2/M cell cycle arrest and survival of irradiated leukemia cells. *Cell Physiol. Biochem.* 26 (4-5), 597–608. doi: 10.1159/000322327

Huber, S. M., Misovic, M., Mayer, C., Rodemann, H. P., and Dittmann, K. (2012). EGFR-mediated stimulation of sodium/glucose cotransport promotes survival of irradiated human A549 lung adenocarcinoma cells. *Radiother. Oncol.* 103 (3), 373–379. doi: 10.1016/j.radonc.2012.03.008

Huber, S. M. (2013). Oncochannels. *Cell Calcium* 53 (4), 241–255. doi: 10.1016/j.ceca.2013.01.001

Huber, S. M., Butz, L., Stegen, B., Klumpp, D., Braun, N., Ruth, P., et al. (2013). Ionizing radiation, ion transports, and radioresistance of cancer cells. *Front. Physiol.* 4, 212. doi: 10.3389/fphys.2013.00212

Klumpp, D., Misovic, M., Szteyn, K., Shumilina, E., Rudner, J., and Huber, S. M. (2016). Targeting TRPM2 Channels Impairs Radiation-Induced Cell Cycle Arrest and Fosters Cell Death of T Cell Leukemia Cells in a Bcl-2-Dependent Manner. *Oxid. Med. Cell Longev.* 2016, 8026702. doi: 10.1155/2016/8026702

Klumpp, D., Frank, S. C., Klumpp, L., Sezgin, E. C., Eckert, M., Edalat, L., et al. (2017). TRPM8 is required for survival and radioresistance of glioblastoma cells. *Oncotarget* 8 (56), 95896–95913. doi: 10.18632/oncotarget.21436

Klumpp, L., Sezgin, E. C., Skardelly, M., Eckert, F., and Huber, S. M. (2018). KCa3.1 Channels and Glioblastoma: In Vitro Studies. *Curr. Neuropharmacol.* 16 (5), 627–635. doi: 10.2174/1570159x15666170808115821

Larsen, A. P., Olesen, S. P., Grunnet, M., and Jespersen, T. (2008). Characterization of hERG1a and hERG1b potassium channels-a possible role for hERG1b in the I (Kr) current. *Pflugers Arch.* 456 (6), 1137–1148. doi: 10.1007/s00424-008-0476-7

Lehmann, D. F., Eggleston, W. D., and Wang, D. (2018). Validation and Clinical Utility of the hERG IC50:Cmax Ratio to Determine the Risk of Drug-Induced Torsades de Pointes: A Meta-Analysis. *Pharmacotherapy* 38 (3), 341–348. doi: 10.1002/phar.2087

Li, H., Liu, L., Guo, L., Zhang, J., Du, W., Li, X., et al. (2008). HERG K+ channel expression in CD34+/CD38-/CD123(high) cells and primary leukemia cells and analysis of its regulation in leukemia cells. *Int. J. Hematol.* 87 (4), 387–392. doi: 10.1007/s12185-008-0056-9

Manoli, S., Coppola, S., Duranti, C., Lulli, M., Magni, L., Kuppalu, N., et al. (2019). The Activity of Kv 11.1 Potassium Channel Modulates F-Actin Organization During Cell Migration of Pancreatic Ductal Adenocarcinoma Cells. *Cancers (Basel)* 11 (2), 1–18. doi: 10.3390/cancers11020135

Mohr, C. J., Gross, D., Sezgin, E. C., Steudel, F. A., Ruth, P., Huber, S. M., et al. (2019). KCa3.1 Channels Confer Radioresistance to Breast Cancer Cells. *Cancers (Basel)* 11 (9), 1–18. doi: 10.3390/cancers11091285

Palme, D., Misovic, M., Schmid, E., Klumpp, D., Salih, H. R., Rudner, J., et al. (2013). Kv3.4 potassium channel-mediated electrosignaling controls cell cycle and survival of irradiated leukemia cells. *Pflugers Arch.* 465 (8), 1209–1221. doi: 10.1007/s00424-013-1249-5

Pillozzi, S., and Arcangeli, A. (2010). Physical and functional interaction between integrins and hERG1 channels in cancer cells. *Adv. Exp. Med. Biol.* 674, 55–67. doi: 10.1007/978-1-4419-6066-5_6

Pillozzi, S., Masselli, M., De Lorenzo, E., Accordi, B., Cilia, E., Crociani, O., et al. (2011). Chemotherapy resistance in acute lymphoblastic leukemia requires hERG1 channels and is overcome by hERG1 blockers. *Blood* 117 (3), 902–914. doi: 10.1182/blood-2010-01-262691

Pillozzi, S., D'Amico, M., Bartoli, G., Gasparoli, L., Petroni, G., Crociani, O., et al. (2018). The combined activation of KCa3.1 and inhibition of Kv11.1/hERG1 currents contribute to overcome Cisplatin resistance in colorectal cancer cells. *Br. J. Cancer* 118 (2), 200–212. doi: 10.1038/bjc.2017.392

Pointer, K. B., Clark, P. A., Eliceiri, K. W., Salamat, M. S., Robertson, G. A., and Kuo, J. S. (2017). Administration of Non-Torsadogenic human Ether-a-go-go-Related Gene Inhibitors Is Associated with Better Survival for High hERG-Expressing Glioblastoma Patients. *Clin. Cancer Res.* 23 (1), 73–80. doi: 10.1158/1078-0432.Ccr-15-3169

Polak, S., Wisniowska, B., and Brandys, J. (2009). Collation, assessment and analysis of literature in vitro data on hERG receptor blocking potency for subsequent modeling of drugs' cardiotoxic properties. *J. Appl. Toxicol.* 29 (3), 183–206. doi: 10.1002/jat.1395

Proudfoot, J. M., Murrey, M. W., McLean, S., Greenland, E. L., Barden, A. E., Croft, K. D., et al. (2018). F2-isoprostanes affect macrophage migration and CSF-1 signalling. *Free Radic. Biol. Med.* 126, 142–152. doi: 10.1016/j.freeradbiomed.2018.08.007

Roth, B., Gibhardt, C. S., Becker, P., Gebhardt, M., Knoop, J., Fournier, C., et al. (2015). Low-dose photon irradiation alters cell differentiation via activation of hIK channels. *Pflugers Arch.* 467 (8), 1835–1849. doi: 10.1007/s00424-014-1601-4

Skelding, K. A., Rostas, J. A., and Verrills, N. M. (2011). Controlling the cell cycle: the role of calcium/calmodulin-stimulated protein kinases I and II. *Cell Cycle* 10 (4), 631–639. doi: 10.4161/cc.10.4.14798

Smith, G. A., Tsui, H. W., Newell, E. W., Jiang, X., Zhu, X. P., Tsui, F. W., et al. (2002). Functional up-regulation of HERG K+ channels in neoplastic hematopoietic cells. *J. Biol. Chem.* 277 (21), 18528–18534. doi: 10.1074/jbc.M200592200

Stegen, B., Butz, L., Klumpp, L., Zips, D., Dittmann, K., Ruth, P., et al. (2015). Ca2+-Activated IK K+ Channel Blockade Radiosensitizes Glioblastoma Cells. *Mol. Cancer Res.* 13 (9), 1283–1295. doi: 10.1158/1541-7786.Mcr-15-0075

Stegen, B., Klumpp, L., Misovic, M., Edalat, L., Eckert, M., Klumpp, D., et al. (2016). K(+) channel signaling in irradiated tumor cells. *Eur. Biophys. J.* 45 (7), 585–598. doi: 10.1007/s00249-016-1136-z

Steinle, M., Palme, D., Misovic, M., Rudner, J., Dittmann, K., Lukowski, R., et al. (2011). Ionizing radiation induces migration of glioblastoma cells by activating BK K(+) channels. *Radiother. Oncol.* 101 (1), 122–126. doi: 10.1016/j.radonc.2011.05.069

Swulius, M. T., and Waxham, M. N. (2008). Ca(2+)/calmodulin-dependent protein kinases. *Cell Mol. Life Sci.* 65 (17), 2637–2657. doi: 10.1007/s00018-008-8086-2

Vandenberg, J. I., Perry, M. D., Perrin, M. J., Mann, S. A., Ke, Y., and Hill, A. P. (2012). hERG K(+) channels: structure, function, and clinical significance. *Physiol. Rev.* 92 (3), 1393–1478. doi: 10.1152/physrev.00036.2011

Voos, P., Fuck, S., Weipert, F., Babel, L., Tandl, D., Meckel, T., et al. (2018). Ionizing Radiation Induces Morphological Changes and Immunological Modulation of Jurkat Cells. *Front. Immunol.* 9, 922. doi: 10.3389/fimmu.2018.00922

Wang, H., Zhang, Y., Cao, L., Han, H., Wang, J., Yang, B., et al. (2002). HERG K+ channel, a regulator of tumor cell apoptosis and proliferation. *Cancer Res.* 62 (17), 4843–4848.

Webster, R., Allan, G., Anto-Awuakye, K., Harrison, A., Kidd, T., Leishman, D., et al. (2001). Pharmacokinetic/pharmacodynamic assessment of the effects of E4031, cisapride, terfenadine and terodiline on monophasic action potential duration in dog. *Xenobiotica* 31 (8-9), 633–650. doi: 10.1080/00498250110054632

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 Palme, Misovic, Ganser, Klumpp, Salih, Zips and Huber. 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 Pharmacology

ORIGINAL RESEARCH
published: 08 May 2020
doi: 10.3389/fphar.2020.00577

Repurposing Kir6/SUR2 Channel Activator Minoxidil to Arrests Growth of Gynecologic Cancers

*Daniela Fukushiro-Lopes[1], Alexandra D. Hegel[1,2], Angela Russo[3], Vitalyi Senyuk[2], Margaret Liotta[4], Gyda C. Beeson[5], Craig C. Beeson[5], Joanna Burdette[3], Ronald K. Potkul[4] and Saverio Gentile[1,2]**

[1] Department of Pharmacology, Loyola University Chicago, Maywood, IL, United States, [2] Department of Medicine, University of Illinois Chicago, Chicago, IL, United States, [3] Department of Pharmaceutical Sciences, College of Pharmacy, University of Illinois Chicago, Chicago, IL, United States, [4] Department of Gynecologic Oncology, Loyola University Chicago, Maywood, IL, United States, [5] Department of Drug Discovery and Biomedical Sciences, Medical University of South Carolina, Charleston, SC, United States

OPEN ACCESS

Edited by:
Sébastien Roger, Université de Tours, France

Reviewed by:
Md Kamal Hossain, University of Michigan, United States
Albrecht Schwab, University of Münster, Germany

***Correspondence:**
Saverio Gentile
sgentile@uic.edu

Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Pharmacology

Received: *06 December 2019*
Accepted: *15 April 2020*
Published: *08 May 2020*

Citation:
Fukushiro-Lopes D, Hegel AD, Russo A, Senyuk V, Liotta M, Beeson GC, Beeson CC, Burdette J, Potkul RK and Gentile S (2020) Repurposing Kir6/SUR2 Channel Activator Minoxidil to Arrests Growth of Gynecologic Cancers. Front. Pharmacol. 11:577. doi: 10.3389/fphar.2020.00577

Gynecologic cancers are among the most lethal cancers found in women, and, advanced stage cancers are still a treatment challenge. Ion channels are known to contribute to cellular homeostasis in all cells and mounting evidence indicates that ion channels could be considered potential therapeutic targets against cancer. Nevertheless, the pharmacologic effect of targeting ion channels in cancer is still understudied. We found that the expression of Kir6.2/SUR2 potassium channel is a potential favorable prognostic factor in gynecologic cancers. Also, pharmacological stimulation of the Kir6.2/SUR2 channel activity with the selective activator molecule minoxidil arrests tumor growth in a xenograft model of ovarian cancer. Investigation on the mechanism linking the Kir6.2/SUR2 to tumor growth revealed that minoxidil alters the metabolic and oxidative state of cancer cells by producing mitochondrial disruption and extensive DNA damage. Consequently, application of minoxidil results in activation of a caspase-3 independent cell death pathway. Our data show that repurposing of FDA approved K^+ channel activators may represent a novel, safe adjuvant therapeutic approach to traditional chemotherapy for the treatment of gynecologic cancers.

Keywords: ion channels, cancer treatment, repurposing drug, minoxidil, oxidative stress, mitochondria

INTRODUCTION

Gynecologic cancer is an uncontrolled growth and spread of malignant cells arising from the female reproductive tract. Although gynecologic cancers are a leading cause of death worldwide, they are under-studied. Endometrial cancer is the most common cancer of the female reproductive system for which the death rate has increased more than 100% in the past two decades. Whereas, ovarian/fallopian tube cancer, which mostly present the high-grade serous carcinoma (HGSC) histology type is the most lethal with a 5-year survival rate of only 30% and, more than 140,000 women die each year (Ries and Klastersky, 1986; Tomita et al., 2001; Miller et al., 2010; Zhang et al., 2014) worldwide

because of disease progression. Also, ovarian cancer is a significant contributor to cancer disparities, since African-American women die at almost twice the rate as other racial groups (Mannhold, 2004).

Failure to treat these cancers is related to a variety of causes including late diagnosis and cancer heterogeneity. Also, the dose-limiting toxicity of available therapeutic agents can increase the morbidity and limits the ability to deliver the optimal therapeutic dose. Recently, targeted therapy has been evaluated to overcome resistant ovarian cancer but the lack of targets and the narrow therapeutic window results in an unfavorable risk/benefit profile. Ultimately, lack of population diversity on genomic database poses a serious limitation to translate research into practice (Landry et al., 2018). Consequently, it is important to identify novel targets and approaches to treat gynecologic cancers.

Potassium (K^+) ion channels are integral membrane proteins that selectively allow an outflow of K^+ ion to cross the cellular membrane. K^+ channels have been traditionally studied for their role in controlling neuronal transmission, muscle contraction, or secretion. Nevertheless, recently studies have demonstrated that several members of the K^+ channel family play a fundamental role in governing other critical cellular events such as proliferation (Wang, 2004; Beech and Cheong, 2006; Urrego et al., 2014; Bull and Doig, 2015; Rao et al., 2015; Gentile, 2016). Remarkably, cancers of different histotypes present alteration of specific K^+ ion channel expression suggesting that these proteins could be critical factors in cancer biology.

The Kir6/SUR potassium channel (**Figure 1A**) comprises a tetrameric structure in which four identical subunits Kir6.1 or Kir6.2 (encoded by the ABCC8 and ABCC9 genes respectively) are in complex with the sulfonylurea receptor SUR1 or SUR2 (encoded by the KCNJ8 and KCNJ11 genes respectively). While the Kir6 protein forms the channel pore, the SUR subunit is essential for controlling the opening and activity of the channel (Campbell et al., 2003; Mannhold, 2004). The presence of Kir6 subunit alone constitutes a non-functional channel.

Recent studies have revealed that the SUR subunit controls the selectivity of the pharmacological response to the drug that either inhibit or stimulate the Kir6/SUR channel. For example, glibenclamide (Glynase ®) acts as anti-diabetic medicine (type II diabetic patients) by inhibiting Kir6.1/SUR1 channel activity upon direct binding to the SUR1 subunit. Furthermore, minoxidil acts as an activator of the Kir6/SUR2 channel upon selective binding to SUR2 and does not generate significant side effects even at high doses. Minoxidil is approved by the FDA as anti-hypertensive agent (Loniten ®) and to arrest hair loss (Rogaine ®) (Bolduc and Shapiro, 2000; Rivera and Guerra-Tapia, 2008; Shorter et al., 2008). Remarkably, expression of different subunits of the Kir6/SUR complex presents tissue specificity. For example, neurons and pancreatic β-cells can express any of the Kir6 with SUR1 (but not SUR2) that contributes to electrical transmission or insulin secretion. In contrast, cardiac myocytes and vascular smooth muscle can express any of the Kir6 with SUR2 that contribute to protection against metabolic stress (Sato et al., 2004). Therefore, tissue specificity is a major contributor for the efficacy and safety (lack of significant side effects) of molecules that target SUR1 (e.g. Glynase) rather than SUR2 (e.g. Loniten).

Although drug discovery focusing on ion channels has generated an abundance of medicines of critical importance for treating human diseases, (Bull and Doig, 2015) the benefit of targeting the K^+ channel in cancer has not yet been considered thoroughly. In our previous work, we have demonstrated that use of K^+ channel activators can affect several hallmarks of cancer (Lansu and Gentile, 2013; Perez-Neut et al., 2015; Perez-Neut et al., 2016; Fukushiro-Lopes et al., 2018). In this study we found that gynecologic cancers express the Kir6.2/SUR2 K^+ channel. We demonstrate that stimulation of the Kir6.2/SUR2 activity with the pharmacological activator Minoxidil (Loniten ®) produces cell death in endometrial and ovarian cancer cells which then translates into reduced tumor growth.

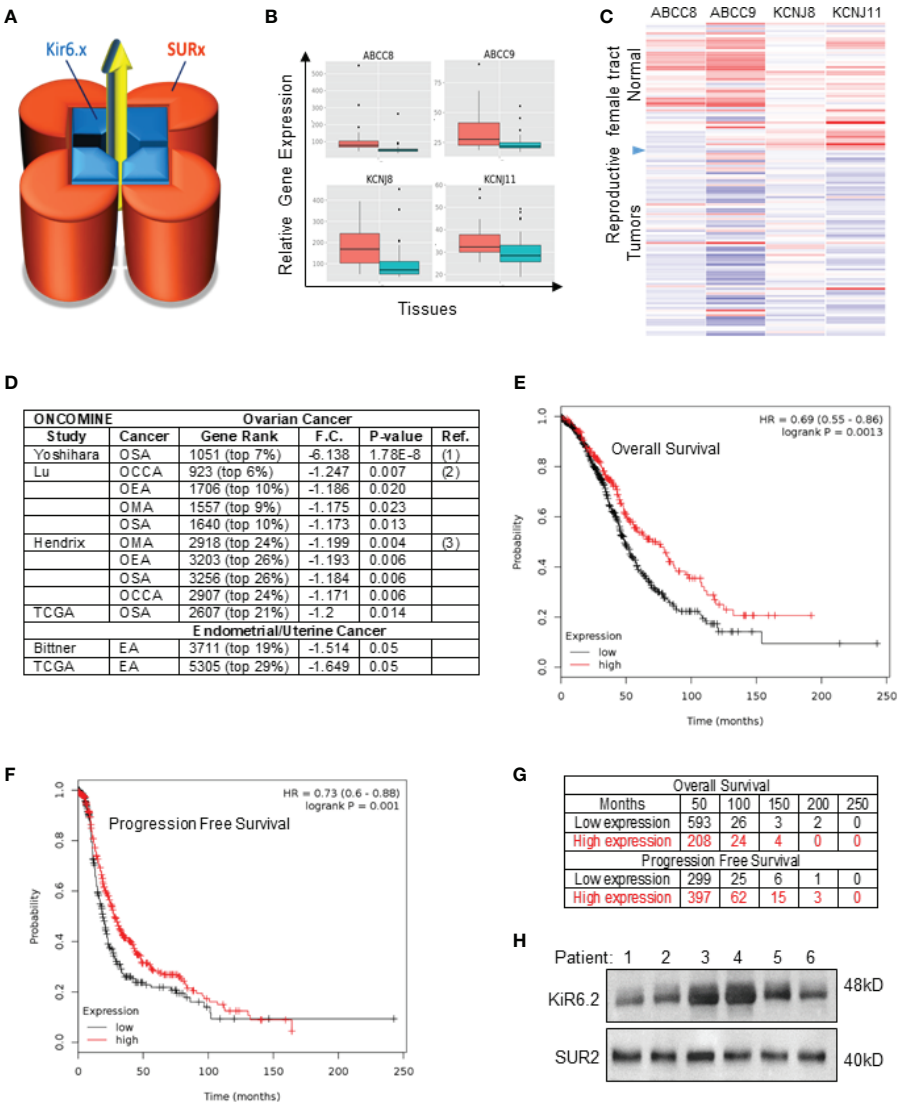
RESULTS

Kir6.2/SUR2 Channel Is a Potential Favorable Prognostic Factor in Ovarian Cancer

In order to first determine the expression levels of potassium channel genes in gynecological cancers, a bioinformatic investigation was completed with the merav.wi.mit.edu (Shaul et al., 2016), Oncomine.org (Rhodes et al., 2004) which are curated gene expression database of publicly available microarray datasets. This study revealed that the KCNJ8, KCNJ11, ABCC8, ABCC9 genes are downregulated in cancer of the female reproductive tract when compared with the correspondent normal tissues (**Figures 1B, C**).

Interestingly, a differential expression analyses revealed that the ABCC9 (SUR2) gene is significantly downregulated in human ovarian cancer tissues independently of their histological characterization and in uterine cancer (**Figures 1C, D**). Analyses of the TCGA database showed that the SUR2 subunit appeared to be downregulated in at least 82% of the HGSC compared to healthy tissue (**Supplementary Figure 1B**). In contrast, KCNJ11 or KCNJ8 gene copy was unaltered.

To explore the clinical relevance of our findings, we conducted *in silico* analysis with the Kaplan-Meier Plotter database (KM plotter.com) (Nagy et al., 2018) by performing survival analyses based on selection of SUR2 as biomarker expression levels. This investigation revealed that high expression of the SUR2 gene is associated with improved overall survival (OS) in all ovarian cancer patients [Hazard Ratio (HR)= 0.7 (0.55-0.86); **Figures 1E–G**] with a 49% reduction in mortality and improved progression-free survival PFS [HR=0.73 (0.6–0.88); **Figure 1H**]. Subgroup analysis revealed that OS further improves in patients diagnosed with stage IV ovarian cancer [HR= 0.64 (0.5-0.84); **Supplemental Figure 1C**]. To further validate our bioinformatics study, we monitored protein expression level of the ion channels and functional subunits on ovarian cancer samples obtained from patient tissue donors that were diagnosed with stage IV high



ONCOMINE		Ovarian Cancer			
Study	Cancer	Gene Rank	F.C.	P-value	Ref.
Yoshihara	OSA	1051 (top 7%)	-6.138	1.78E-8	(1)
Lu	OCCA	923 (top 6%)	-1.247	0.007	(2)
	OEA	1706 (top 10%)	-1.186	0.020	
	OMA	1557 (top 9%)	-1.175	0.023	
	OSA	1640 (top 10%)	-1.173	0.013	
Hendrix	OMA	2918 (top 24%)	-1.199	0.004	(3)
	OEA	3203 (top 26%)	-1.193	0.006	
	OSA	3256 (top 26%)	-1.184	0.006	
	OCCA	2907 (top 24%)	-1.171	0.006	
TCGA	OSA	2607 (top 21%)	-1.2	0.014	
Endometrial/Uterine Cancer					
Bittner	EA	3711 (top 19%)	-1.514	0.05	
TCGA	EA	5305 (top 29%)	-1.649	0.05	

Overall Survival					
Months	50	100	150	200	250
Low expression	593	26	3	2	0
High expression	208	24	4	0	0
Progression Free Survival					
Low expression	299	25	6	1	0
High expression	397	62	15	3	0

FIGURE 1 | SUR2 expression and survival in patients with ovarian cancer. **(A)** Schematic representation of the Kir6/SUR potassium channel complex. **(B)** Boxplot and **(C)** Heat map representing analyses of copy number alteration for ABCC8, ABCC9, KCNJ8 and KCNJ11 genes in human primary tumors (■) and normal tissues (■) of the female reproductive system (merav.wi.mit.edu; criteria: p<0.5; fold change > 1.5). **(D)** Western blot showing expression of Kir6.2 and SUR2 proteins in 6 representative ovarian cancer tissues obtained from patients (Stage IV; IRB: LU209031, Loyola University Chicago) and **(E)** in SPEC-2, OVCAR-8 or OVCAR-4 human derived cell lines. **(F)** List of studies showing downregulation of the SUR2 gene in ovarian cancers compared to normal tissue (Oncomine.org dataset; threshold by: P-Value: 1–4; Fold Change: 2; Gene rank: Top 10%.). OSA, Ovarian Serous Adenocarcinoma; OCCA, Ovarian Clear Cell Adenocarcinoma; OEA, Ovarian Endometrioid Adenocarcinoma; OMA, Ovarian Mucinous Adenocarcinoma; Gene ranking, fold change (F.C.), p-value and probe used are reported. **(G)** Kaplan–Meier plots of overall survival and **(H)** progression-free survival in patients with ovarian cancer comparing the patients with high (red) and low (black) expression of SUR2 (top vs. bottom tertiles). For OS the median survival in low expression cohort = 49 months. Hazard ratios (HR) compare the hazard of relapse or death in the high expression versus low expression groups. Probe: Affy ID: 208462_s_at; Datasets: GSE14764, GSE23554, GSE26193, GSE26712, GSE30161, GSE3149, GSE32062, GSE63885, GSE9891, TCGA.

grade serous cancer. This investigation revealed that both Kir6.2 (KCNJ11) and SUR2 (ABCC9) proteins can be expressed in ovarian cancer (**Figure 1H**). Our data suggest that the KCNJ11 and SUR2 genes could be considered as a potential prognostic factor. Also, ovarian cancer patients could benefit from the pharmacological stimulation of the Kir6.2/SUR2 channel activity by minoxidil.

Minoxidil Arrest Ovarian Cancer Tumor Growth

To evaluate the relevance of our bioinformatics investigation, we established a xenograft model of Kir6.2/SUR2 positive HGSC cell line (**Supplementary Figure 1**) in the NOD-SCID-IL2Rγnull (NSG) (Villanueva et al., 2013) mice in which we assessed the effect of minoxidil (**Figure 2**). As expected, 6 weeks after cell

implant, all control mice (untreated) presented ascites fluid (mean volume of 2.8 ml). Dissection of the control mice revealed that all mice presented a primary tumor and hundreds of tumor nodules that had developed on the peritoneal wall. In contrast, in five of the six mice that were treated with minoxidil, no measurable amount of ascites, tumor or metastasis were produced (10mg/Kg; **Figures 2A, B**). Mice treated with higher dose of minoxidil (50 mg/Kg) were severely stressed after four injections as they presented limited motility, inappetence, loss of weight and lethargic behavior and were sacrificed before the end of the study. The total weight of the only primary tumor found in the minoxidil treated group was estimated to be decreased of 90-fold when compared with the tumor of the untreated mouse. These experiments reveal that the Kir6.2/SUR channel activator minoxidil inhibited ovarian tumor development.

Minoxidil Does Not Alter Cardiac Function in NSG Mouse Harboring Ovarian Cancer

Cardiotoxicity related to drugs that are used during anticancer treatments can often limit the benefit of anticancer therapy and can cause interruption of the treatment. Minoxidil is considered a safe drug as it has been widely used both topically (Rogaine ®) and systemically (Loniten ®) in humans worldwide without generating significant side effects (Pettinger, 2017). Nevertheless, minoxidil has never been tested as a therapeutic agent against ovarian cancer and the potential cardiotoxicity of minoxidil in cancer patients is unknown. To address this potential issue, we used transthoracic echocardiograms (TTA) to evaluate cardiac performance in mice baring ovarian cancer (**Figure 3**). TTA was performed within 2 h or 25 days after exposure to minoxidil to measure respectively acute and chronic response to the drug.

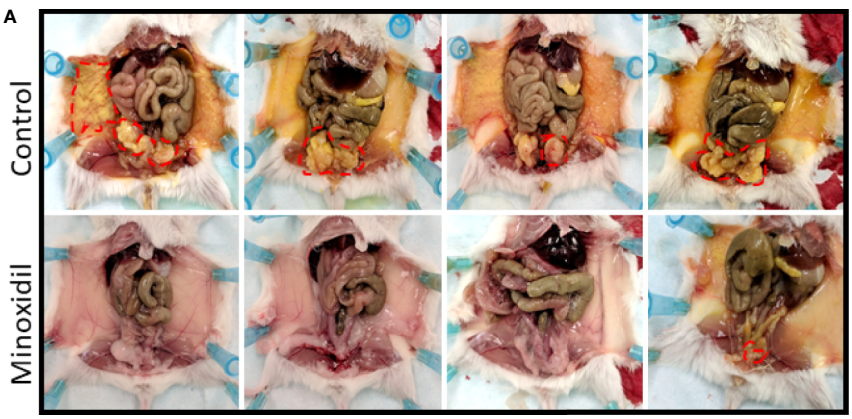
Notably, we found that minoxidil did not produce a change in any of the parameters measured suggesting that minoxidil can be considered a safe antiproliferative agents against ovarian cancer.

Minoxidil Inhibits Proliferation and Arrests the Cell Cycle

To understand the mechanism that underlies the inhibitory effect of minoxidil on ovarian tumor growth, we monitored the effects of minoxidil on proliferation of Kir6.2/SUR2 positive or Kir6.2/SUR2 negative cell lines (**Figure 4A** and **Supplementary Figure 1**).

We found that application of minoxidil (24 h) strongly inhibited proliferation rate in OVCAR-8 and SPEC-2 cell lines in a concentration-dependent manner (**Figure 4A**). In contrast, proliferation OVCAR-4 which do not express SUR2 was not affected by minoxidil at any concentration (**Figure 4A**). To gain insights on the specificity of minoxidil we produced an OVCAR-8 cell line in which expression of SUR2 was suppressed by si-RNA (siSUR2-OVCAR-8). As expected, minoxidil did not significantly alter the proliferation rate of these cells indicating

A



B

	Ascites	Tumor	Metastasis
Control	6	6	6
Minoxidil	0	1	0

FIGURE 2 | Effect of minoxidil on HGSC ovarian tumor development. **(A)** Representative mice with tumor burdens (red circle) treated with vehicle alone (Control) or with minoxidil (10 mg/kg). OVCAR8 cells were injected intraperitoneally into 12 female NSG mice (7 weeks old). Mice were separated randomly in two groups of six mice each. Two weeks after cell implant, one group of six mice was injected (IP; twice/wk) with 100 µl vehicle alone (DMSO) or minoxidil. White arrows indicate the presence of tumors. **(B)** Table listing the number of mice presenting ascites, tumor or metastasis (tumor formation in the peritoneum) in the control or treated groups.

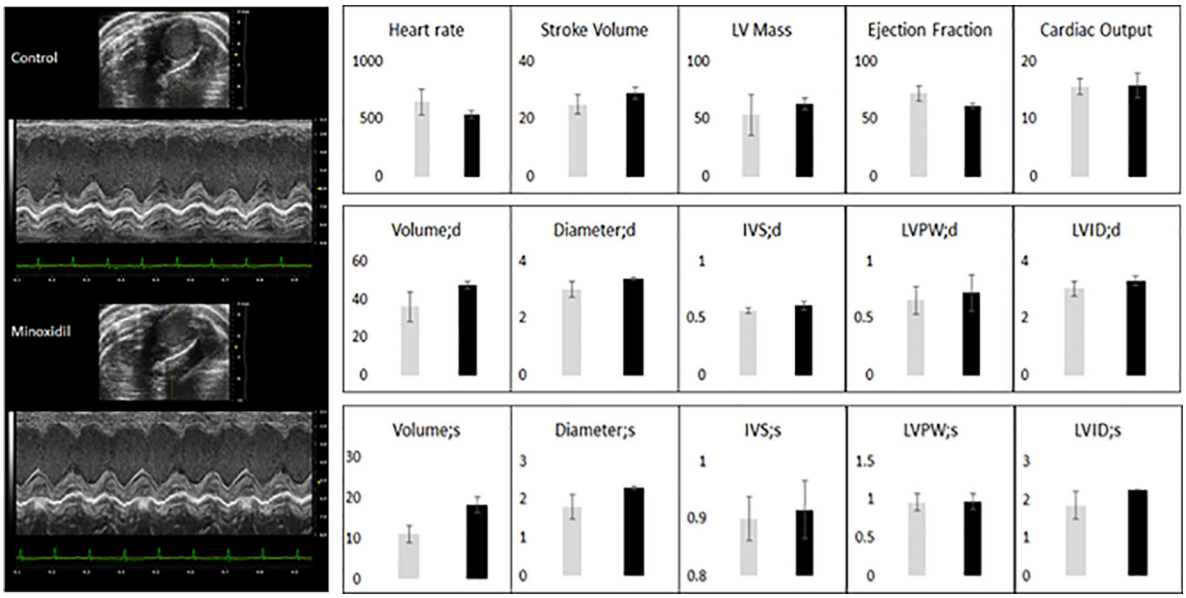


FIGURE 3 | Effect of minoxidil on *in vivo* cardiac function. Transthoracic M-mode echocardiograms echocardiography (Vevo 2100 with MS400 series transducer, Visualsonics Inc.) of (A) control (n = 3) and minoxidil-treated mice (n = 3). Figure represents a motion mode of the left ventricle, which is obtained with a single ultrasound beam transmitted through the heart with the resulting image displayed over time. Light Gray bar = control; Dark gray bar = minoxidil. HR, heart rate (beats per min); EF, ejection fraction (%); LVIDs & LVIDd, left ventricular internal diameter systole and diastole; LVAWs & LVAWd, left ventricular posterior wall systole and diastole (mm). Beat-to-beat detection was achieved using preset detection and analysis settings for mice (typical QRS width 10 ms, R waves > 60 ms apart, Pre-P baseline 10 ms, Maximum PR 50 ms, Maximum RT 40 ms, ST height at 10 ms, averaging four beats). Averaged ECG measurements were taken from a minimum of 20 beats per mouse. ECG analysis was performed in LabChart8 (ADInstruments).

that the inhibitory effect of minoxidil is mediated specifically by SUR2.

To better characterize the mechanism through which minoxidil suppresses ovarian tumor growth, we monitored changes in the cell cycle with flow cytometry (**Figure 4B**). Kir6.2/SUR2 positive or Kir6.2/SUR2 negative cell lines were treated with minoxidil or DMSO (control) for 24 h. Next, DNA content in cells from each plate was analyzed with flow cytometry by propidium iodide staining. We found that in both Kir6.2/SUR2 positive cell lines (OVCAR-8 and SPEC2) minoxidil produced a depletion of cells in G0/G1 and in G2/M phase and, an accumulation of cells in S phase of the cell cycle in a dose-dependent manner (**Figures 4B–D**). In contrast, in the Kir6.2/SUR2 negative cell line OVCAR-4, minoxidil did not produce any significant changes in the distribution of cells in different cell cycle phases (**Supplementary Figure 1D**)

It has been established that the entry of cells into mitosis is regulated by important factors such as CDK1 and WEE1 kinases and by cyclin B. Remarkably, we found that minoxidil inhibited CDK1 activity as indicated by the increased phosphorylation of Y15 on CDK1 (**Supplementary Figure 1E**), inhibited WEE1 activity as indicated by reduced phosphorylation of S642 on WEE1 and produced the accumulation of cyclin B.

Our experiments indicate that pharmacological stimulation of the Kir6.2/SUR2 potassium chanel activity with minoxidil inhibited cell proliferation by arresting the cell cycle in the G2/M phase.

Minoxidil Alters Oxidative State by Affecting Mitochondria

We have previously demonstrated that stimulation of a K^+ channel activity in cancer cells can alter the cellular oxidative state in a Ca^{2+} entry-dependent manner. Hence, to gain insight into the Kir6.2/SUR2-dependent mechanism of ovarian cancer growth arrest, we monitored the reactive oxygen species (ROS) level in cells treated with minoxidil. We found that minoxidil significantly increased ROS concentration (**Figure 5A**) when compared to untreated cells. In contrast, application of the Kir6 channels blocker Glibenclamide completely abated the effect of minoxidil on ROS production (**Figure 5A**) confirming that the effects of minoxidil is mediated by Kir6 currents. Also, application of the cell impermeable Ca^{2+} chelator, EGTA to the growth medium suppressed the effect of minoxidil on ROS production (**Figure 5A**) indicating that minoxidil-dependent ROS production is mediated by extracellular Ca^{2+}.

ROS production are generally associated with stressed mitochondria and it underlies oxidative damage that can lead to cell death. Remarkably, we found that use of the specific mitochondria superoxide scavenger, MitoTEMPO completely inhibited the minoxidil-dependent ROS production (**Figure 5B**) and cell death (**Supplementary Figure 2A**). These data suggest that minoxidil alters the cellular oxidative state by affecting mitochondria. To further test this hypothesis, we performed a Mito Stress Test assay with a Seahorse Analyzer with cells treated with or without minoxidil.

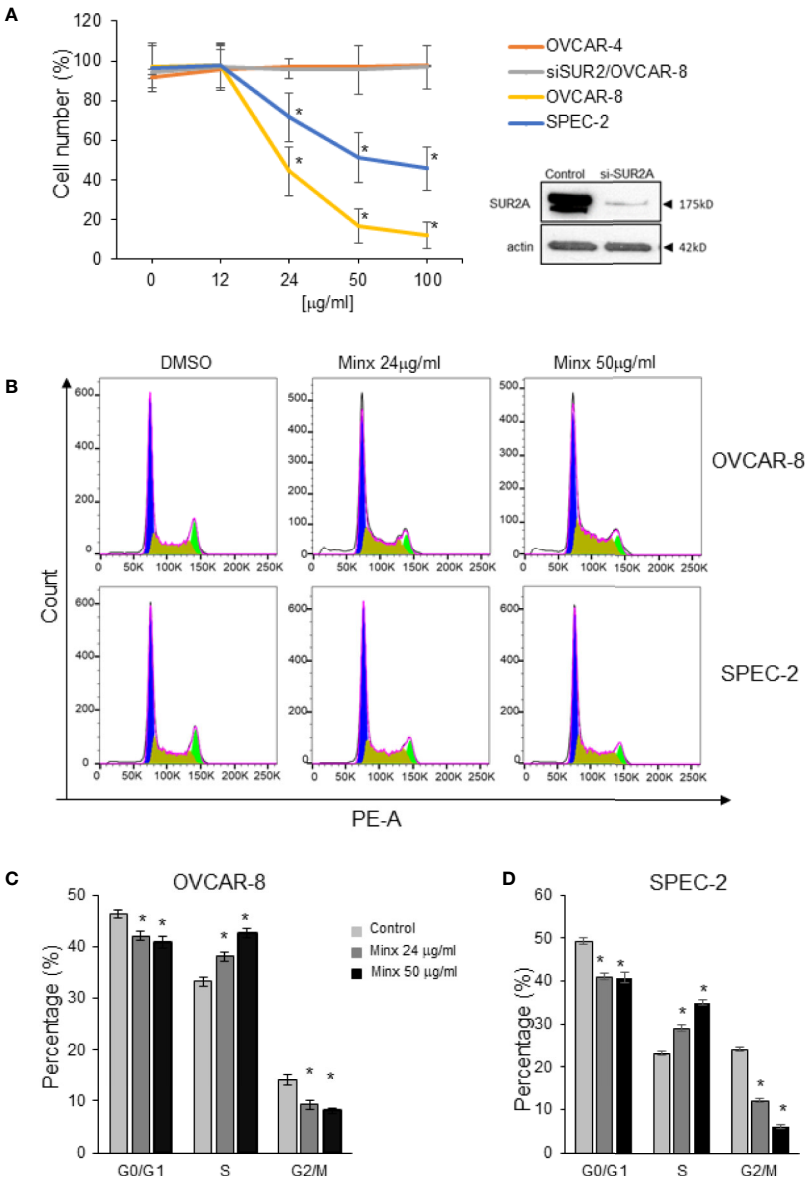


FIGURE 4 | Effect of minoxidil on cell proliferation. **(A)** Percentage of viable cells (MTS assay, Promega) of OVCAR-4, OVCAR-8, OVACR8 with siRNA targeting SUR2 (siSUR2/OVCAR-8), and SPEC-2 exposed to different concentration of minoxidil for 24 h. (To compare data points from different treatment groups, ANOVA and post hoc Dunnett's tests were used. n=6; * P<0.005) and Western blot showing the efficacy of si-RNA in reducing SUR2 expression. **(B)** Representative results showing minoxidil-dependent accumulation in the S phase of the cell cycle of ovarian (OVCAR-8) and uterine (SPEC-2) cancer cell lines. Sub-confluent cells were treated with different concentration of minoxidil or DMSO 24 h and then flow cytometry was performed to analyze cell cycle. **(C, D)** Quantification of the effect of minoxidil on cell cycle Minoxidil. Data represent mean±SEM (n=3; * p<0.001; unpaired t-test).

Interestingly, we found that minoxidil alters mitochondrial function (**Figure 5C**) in a concentrantion-dependent manner. Much of the ATP utilization in cells is committed to maintenance of the Na^+/K^+ gradient via the plasma membrane Na/K-ATPase. The intracellular depletion of K^+ that is caused by activation of Kir6.2/SUR2 via minoxidil is then restored by activation of Na/K-ATPase. This event creates an increased ATP demand while lowering the intracellular K^+ ion concentration and thereby enhancing Ca^{2+} load on the mitochondria.

Collectively these effects should increase basal ATP production via mitochondria as seen in the minoxidil promoted increase in basal oxygen consumption rate (OCR) (**Figure 5C**). Increased flux through the electron transport chain will also increase leak of electrons to oxygen resulting in enhanced formation of superoxide ions. The enhanced leak

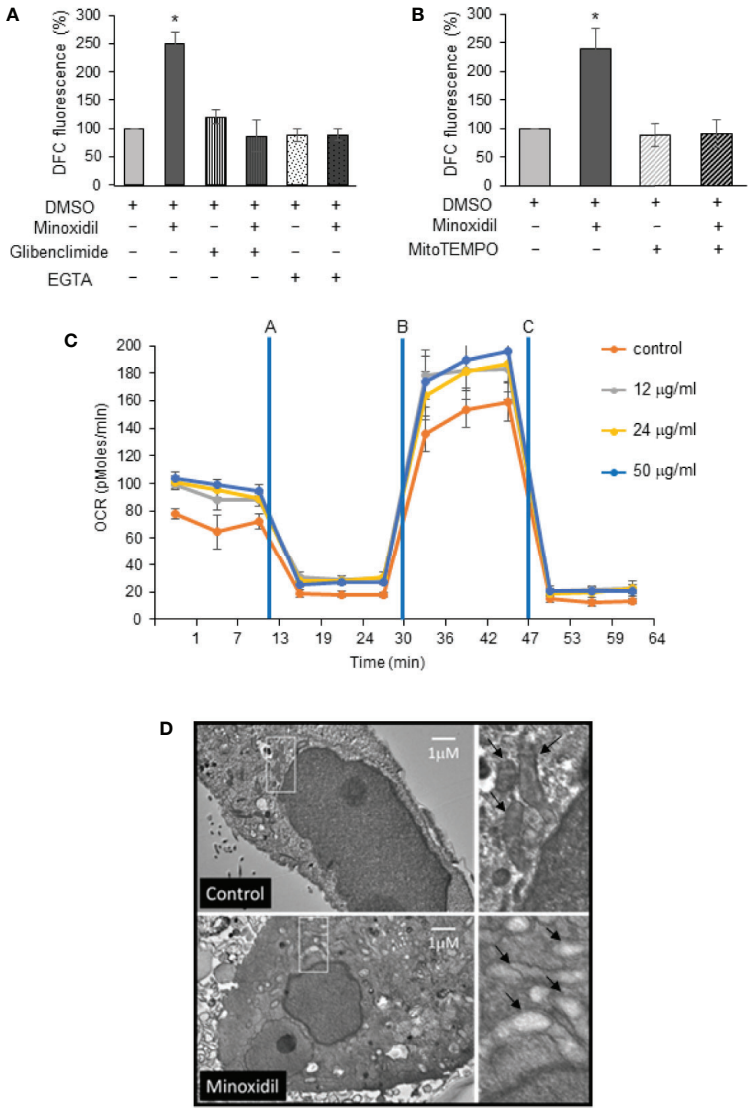


FIGURE 5 | Minoxidil disrupts mitochondria function. **(A)** Effect of minoxidil alone (50 µg/ml) or with the Kir6.2/SUR blocker glibenclimide (10 µg/ml) or the Ca^{2+} ion chelator EGTA (2.4 µg/ml) or **(B)** with MitoTEMPO (5 µg/ml L) compared to control (DMSO) on cellular ROS formation in human-derived OVCAR-8 (DCFH-DA to 2′,7′-dichlorofluorescein DCF, Thermo Fisher Sci; Fluorescence was analyzed in a plate reader (PHERAstar FS, BMG LABTECH) with excitation at 485 nm and emission at 520 nm). Data is expressed as mean ± SEM; *$p < 0.001$. **(C)** Kinetic Oxygen Consumption Rate (OCR) of OVCAR-8 cells to minoxidil at 0, 12, 24, or 50 µg/ml. Cells were plated at 25,000/well in XF24 V7 culture plates. The assay medium was the substrate-free base medium. n=3; (Assay design, data analysis, and file management were performed with Agilent Seahorse Wave Desktop software) **(D)** Representative electron microscopy micrographs showing mitochondria in OVCAR-8 cells treated with minoxidil (50 µg/ml; 24 h). The disruptive effect of the drug is shown as lack of cristae in the mitochondria of treated cells (white box enlarged in right panel).

due to minoxidil exposure is most evident when the cells are uncoupled with the carbonyl cyanide-p-trifluoromethox-phenyl-hydrazon (FCCP), minoxidil-treated cells have a higher OCR, and the higher leak when comparing the oligomycin—Antimycin/Rotenone rates that directly measure leak. Collectively, these results suggest that efflux of K^+ due to minoxidil increases ATP demand, leading to higher leak and, thus, higher superoxide levels as suggested by the ROS dye and TEMPO data.

Also, we caried out an electron microscopy analyses to monitor the effect of minoxidil on mitochondria integrity. We found that cancer cells exposed to minoxidil for 24 h presented a severe mitochondrial morphological abnormality (**Figure 5D**) when compared to untreated cells suggesting that the inhibitory effects of minoxdil on ovarian cancer growth is mediated by alteration of mitchocondria integrity.

A major consequence of increased ROS production is alteration of DNA integrity. Therefore, we investigated the effects of minoxidil

on DNA damage. Our Western blot analyses of OVCAR-8 cells line (**Figure 6A**) and immunohistochemistry examination of the tumors extracted from mice (**Figure 6B**) revealed that minoxidil significantly increased DNA damage as indicated by increased protein level of the DNA damage marker γH2AX. These data were confirmed by a comet assay which indicated that minoxidil produced a significant increse of double breaks DNA damage. In contrast, in cells treated with the Mitochondrial ROS buffer, MITOTEMPO, the effect of minoxidil on DNA damage (**Figure 6C**) was completely suppressed.

These data suggest that pharmacological stimulation of the Kir6.2/SUR2 complex severely affects mitochondria and DNA integrity in ovarian cancer cells.

Minoxidil Produces Caspase-3 Independent Cell Death

To understand whether the effects of minoxidil associated with cell death we performed a bi-parametric cytofluorimetric assay by using a fluorescein isothiocyanate (FITC)-conjugated annexin V (AV) and propidium iodide (PI) protocol. Our experiments revealed that minoxidil induced the increase of the percentage of early (Q3 = AV+/PI-) and late apoptotic cells (Q2=AV+/PI+; **Figures 7A, B**). In contrast, treatment with mitoTEMPO completely abolished the effect of minoxidil on cell death (**Supplementary Figure 2**). However, minoxidil treatment failed to produce activation of the apoptotic executioner caspase-3 as indicated by the lack of cleaved caspase-3 and cleaved PARP and, by the caspase-3 activity ELISA assay (**Figures 8A, B**). As control for caspase-3 activity, a separate group of cells were treated with paclitaxel which produced the expected activation of caspase-3. These results show that minoxidil produces cell death in a caspase-independent manner.

Caspase-3 independent cell death can occurr via accumulation of the apoptosis-inducing factor (AIF) into the nucleus which leads to DNA fragmentation. Thus, we monitored the effect of minoxidil on AIF. Our Western blot analyses of AIF

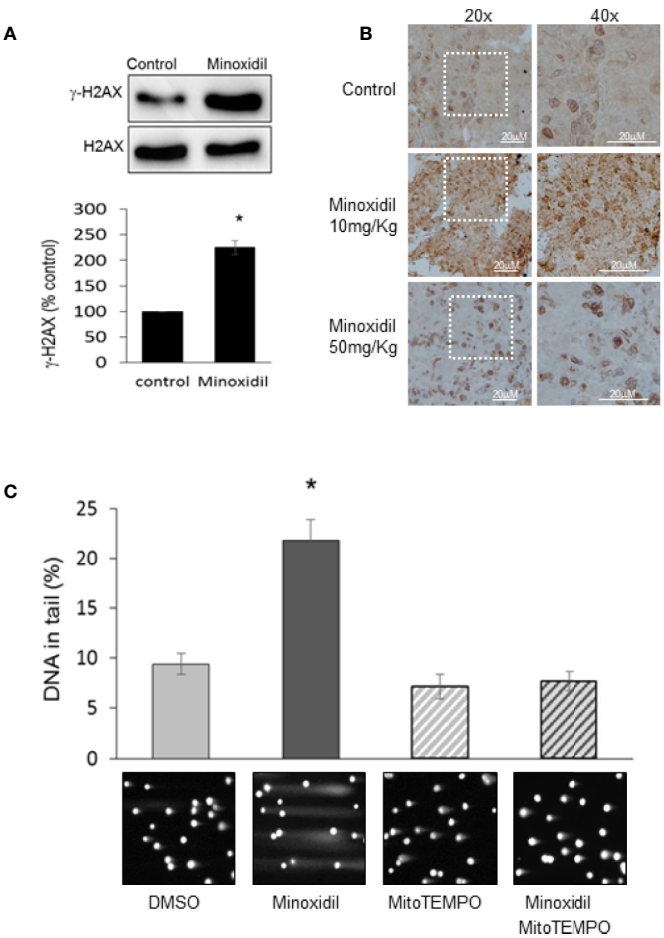


FIGURE 6 | Minoxidil produces DNA damage. **(A)** Western blot analyses of the DNA damage sensor γH2AX in OVCAR-8 cells treated with minoxidil (50 μg/ml) versus control or **(B)** in tumor extracted from mice treated with minoxidil vs control. **(C)** Bar chart representing the effect of minoxidil alone or in combination with MitoTEMPO on DNA integrity. Data is expressed as mean ± SEM; *$p < 0.05$. Bottom panels are representative images of cells subjected to neutral comet assay are presented below the bars corresponding to specific treatments.

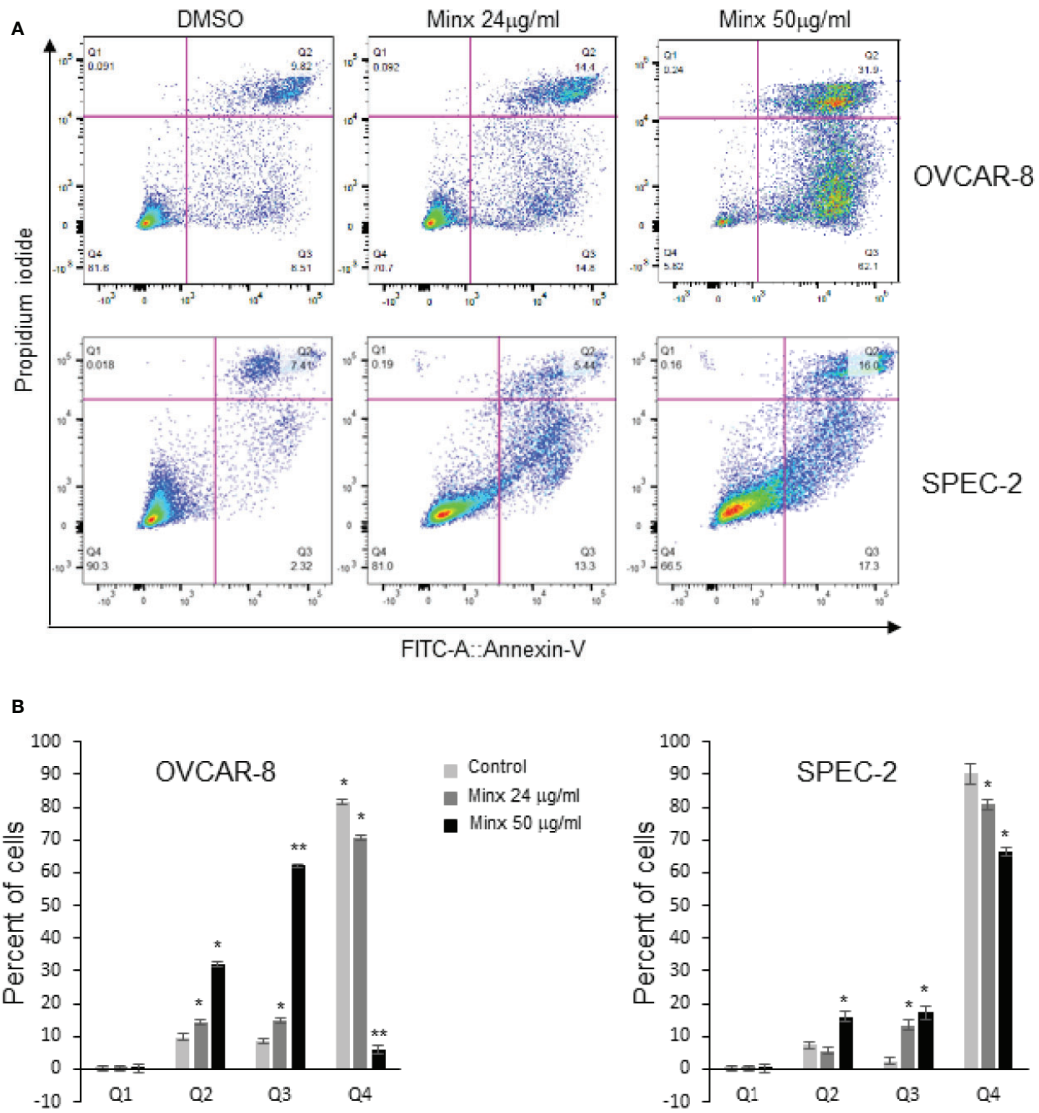


FIGURE 7 | Minoxidil activates cell death. **(A)** Apoptotic analysis of OVCAR-8 or SPEC-2 cells by flow cytometry. Cells were treated with different concentrations of minoxidil alone for 24hr and incubated with AV-FITC and PI. Stained cells were analyzed by Flow cytometry. Percentage of intact cells (AV-/PI–) and different stages apoptotic cells (AV+/PI–, AV+/PI+ and AV-/PI+) are presented. **(B)** Quantification of the apoptotic effect of minoxidil. Data represent mean±SEM (n=3; *p<0.001; ** p<0.0001; unpaired t-test).

expression levels in cell fractions revealed that minoxidil determined a significant increase of AIF protein level in the nucleus (**Figures 8B, C**).

In conclusion, our data demonstrate that the Kir6.2/SUR2 potassium channel could be considered as a potential prognostic factor of cancers cells originated from the female reproductive tract. Also, pharmacological stimulation of the Kir6.2/SUR2 potassium channel activity with the FDA approved molecule minoxidil arrests ovarian tumor growth by arresting the cells in the G2/M phase of the cell cycle. This event associates with alteration of the cellular oxidative state, disruption of the mitochodndria and DNA structure and activatio of a caspase-3 independent cell death.

DISCUSSION

The current study found that selective K^+ channels activators can severely affect viability of ovarian cancer and multiple hallmarks of cancer (Lansu and Gentile, 2013; Perez-Neut et al., 2015; Rao et al., 2015; Fukushiro-Lopes et al., 2018). This suggests that pharmacologically targeting K^+ channels could be considered as a potential anticancer strategy.

Importantly, low expression of the SUR2 gene correlates with poor overall survival in ovarian cancer patients. These data suggest that the SUR2 gene could be a valuable prognostic factor for the most lethal of all gynecologic cancers. Furthermore, ovarian cancer patients could benefit from drugs

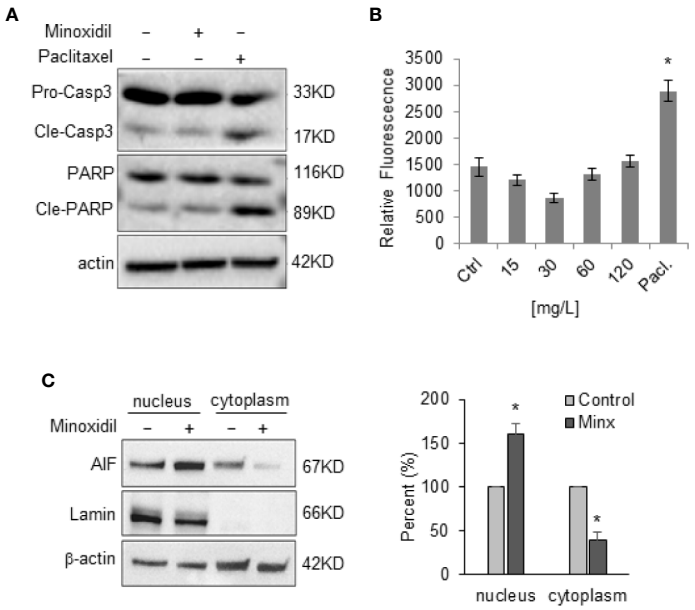


FIGURE 8 | Minoxidil fails to activate casase-3. **(A)** Representative Western blots of caspase-3 and PARP proteins in OVCAR-8 cells treated with minoxidil for 24 h (n=3). The procaspase-3, PARP and their cleaved products are indicated. **(B)** ELISA assay showing the effect of different concentration of minoxidil on caspase activity. Paclitaxel was used as control for caspase activity. To compare data points from different treatment groups, ANOVA and post hoc Dunnett's tests were used. n=3 plates; *p<0.005. **(C)** Western blot analyses of AIF protein level in nuclei and cytoplasm of OVCAR-8 cells treated with minoxidil (50 μg/ml/24 h) and quantification of percent change compared to control *p<0.01.

that stimulate the Kir6.2/SUR2 channel activity and the expressio of the channel may help to stratify patinets for response to the drug. When systemically applied, minoxidil (Loniten ®) is quickly metabolized in minoxidil sulfate, which acts as a selective activator of the Kir6.2 channel upon binding with its constitutive partner SUR2 in the arteries (Mfuh and Larionov, 2015) without producing neurological side effects or insulin disorders due to the absence of the drug target in the brain or pancreas (Mfuh and Larionov, 2015). Experiments demonstrated that application of 10 mg/Kg of minoxidil arrested ovarian tumor growth in NSG mice. In a separate group of NSG mice harboring ovarian tumor, injection of 50 mg/Kg resulted to be toxic.

The dose of minoxidil has been decided based on previously reported experiments demonstrating that 50 mg/Kg body weight (BW) is the maximally tolerated dose in a healthy mouse (Khanna et al., 2011) and on pharmacokinetic studies in humans (Fleishaker and Phillips, 1989). Due to patient-to-patient variation in blood levels, it has been difficult to establish an overdosage level of Loniten ®. At the best of our knowledge, the highest non-lethal blood concentration of minoxidil that has been reported is of ≈3.5 μg/ml (≈3.3 mg/Kg) which correlates to approximately double the maximum suggested dose for anti-hypertensive therapy (100 mg/day; ≈1.6 mg/Kg) (Kikuchi et al., 2016). A recent well-established method to translate the dose of drugs used from one animal species to another has been published (Reagan-Shaw et al., 2008) (http://dtp.nci.nih.gov). On the basis of this formula, the human equivalent dose of 10 mg/Kg of minoxidil in the mouse is 0.8 mg/Kg in a person (average of 60 Kg) which is ≈75% lower than the maximum amount of drug suggested dose of 100 mg/d recommended in the Physician's Desk Reference.

Expression of the Kir6.2/SUR2 channel in the mammalian cardiac myocytes might raise the concern that minoxidil can cause alteration of the heart function. Interestingly, in our experiments we did not observe any significant change of cardiac performance in mice baring ovarian tumors and treated with 10 mg/Kg minoxidil acutely or chronically. Also, previous studies have shown that minoxidil preserves cardiac myocytes by stress damages rather than impinge on cardiac function.

Our in vitro investigation to understand the mechanism underlying the inhibitory effect of minoxidil on ovarian cancer growth revealed that minoxidil significantly altered the production of superoxide species (ROS) in cells. ROS are chemically reactive molecules derived by the reduction of oxigen. Several cellular resources of ROS have been identified but, as mitochondria are actively involved in the production of energy using oxygen, this organelle produce the largest amount of ROS. Ultimately, ROS production is finely controlled by a variety of mechanisms that include changes in intracellular Ca^{2+} concentration and antioxidant activity. Minoxidil significantly increased the oxigen consuption rate (OCR), which was abated by using a ROS scavenger that localizes in the mitochondria. We have previously demonstrated that stimulation of K^+ channel activity in non-excitable cells leads to Ca^{2+} entry which in turn can affect mitochondria-dependent ROS production (Fukushiro-Lopes et al., 2018). As expected, the combination of minoxidil with a Ca^{2+} chelator, which limits the available extracellular Ca^{2+}

to enter the cells, significantly suppressed ROS formation when compared with minoxidili alone. These data show that minoxidil-dependent ROS production is mediated by the mitochondria.

Oxidative cellular stress can occur when the amount of ROS overwhelm the antioxidant mechanisms, which can result in damaging several cellular compartments. Stimulation of the Kir6.2/SUR2 channel with minoxidil produces a catastrophic event that include mitochondria disruption and severe DNA damage. These events associate with activation of a cell-death pathway that is not mediated by the canonical apoptodic executioner caspase-3 or by necrosis as indicated by our annexin V assay. Nonetheless, to better understand that death pathway activated by minoxidil further experiments need to be performed. Also, in addition to the localization at the surface membrane, the minoxidil sensitive Kir/SUR complex has been found expressed in several cell compartments including th sarcoplasmic reticulum (Shorter et al., 2008), endoplasmic reticulum (Salari et al., 2015), mitochondria (Garlid and Halestrap, 2012), and endosome/lysosomes (Bao et al., 2011). Therefore, at this time, we cannot state that the anticancer effect of minoxidil is exclusively mediated by the surface membrane Kir6/SUR2 complex other studies are necessary to confirm our data.

MATERIALS AND METHODS

Tumor Xenograft Models

Two million OVCAR-8 ovarian cancer cells were intraperitoneally (IP) injected in NOD-scid-IL2Rγnull (NSG) female mice (n=12) of 5–6 weeks old. Mice were locally bred and were a generous gift from Michael Nishimura. Mice were randomly divided into two groups (n=6/group) and treated with vehicle (DMSO) or minoxidil via i.p. twice per week. Tumor development was measured every 5 days and mice were sacrificed 6 weeks after the initial minoxidil treatment. This study was reviewed and approved by Loyola University Chicago Institutional Animal Care and Use Committee guidelines (Animal Assurance No. D16-00074). Minoxidil was purchased from Tocris Bioscience [Cat. No. 0583; purity ≥99% (HPLC)].

Effect of Gene Expression Levels on Patient Survival

We performed a meta-analysis to explore the association of *SUR2* gene expression with survival in ovarian cancer patients using KM plotter (KMplotter.org). This web-based application uses a manually curated database that combines all publicly available microarray gene expression data sets. Meta-analyses are performed by integrating gene expression data with clinical data. Patients are segregated into low vs. high expression cohorts, and Kaplan-Meier survival plots with log-rank p values are generated, along with hazard ratios (HR) and 95% confidence intervals (CI). For the meta-analysis of *SUR2* expression, we interrogated the KM plotter database for Affymetrix probe ID: 208462_s_at, and the expression data was partitioned into tertiles. Analyses compared the top and bottom tertile groups.

Cell Culture, Antibodies, and Reagents

OVCAR-4, OVCAR-8 human ovarian cancer cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA). SPEC-2 cells were a generous gift of Dr. Ram Ganapathi (University of North Carolina Charlotte) and maintained 231 cells (ATCC) was maintained in Dulbecco's modified Eagle's medium (DMEM) (4.5 g/L glucose) supplemented with 10% fetal bovine serum (FBS), Penicillin (100 ug/ml)/streptomycin (100 ug/ml) antibiotics. Cells were incubated under humidified conditions with 5% CO_2 at 37°C. All antibodies for Western blot analyses were purchased from Cell Signaling Technologies, Inc (Boston, MA).

Respirometry Assay

OVCAR-8 cells were plated into XF96^e plates at a 50% confluence for 24 h. After the first 24 h the cells were treated with minoxidil (Loniten ®; Rogaine ®) at three different concentrations (0, 60, 120, 240 μM) for the next 24 h. The media was exchanged to a phosphate buffered the assay MEMS modified to contain no bicarbonate and 1 mM phosphate to better measure the acidification rates of the cells. After the cells reached 85% confluence, they were assayed on the XF^{e}96 instrument (Agilent technologies) using a 3 min measure—2 min mix cycle. The oxygen consumption rates (OCR) and extracellular acidification rates were measured with the Mitochondrial Stress Assay, using 1 μM Oligomycin, 1 μM FCCP, and 100 nM Rotenone/100 nM 2 μM Antimycin A. Each treatment was for 15 min during which the OCR and ECAR rates were measured.

Western Blot Analysis, Immunoprecipitation, and Immunofluorescence Staining

Cells were harvested and lysed with cold radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium fluoride (NaF), 1 mM sodium orthovanadate (Na3VO4), and protease inhibitor cocktail]. An equal amount of protein samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. Membranes were blocked with 5% nonfat milk in Tris-Buffered Saline (TBS) containing 0.1% Tween 20 (TBST), incubated with primary and secondary antibodies, and detected using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Pittsburgh, PA). Immunoprecipitation was performed by incubating protein lysates with primary antibodies at 4°C overnight, followed by incubation with protein A/G-conjugated agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). Beads were washed with cold RIPA buffer, resuspended in 2x SDS sample buffer, boiled for 5 min, and subjected to SDS-PAGE, followed by western blot analysis. Cells were grown on poly D-Lysine coated coverslips and treated with or without minoxidil. Following treatment, cells were washed with 1x PBS, fixed with acetone: methanol fixation, blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature, and incubated with primary antibodies, followed by incubation with Alexa Fluor 594- or

Alexa Fluor 488-conjugated secondary antibody (Life Technologies, Carlsbad, CA). Next, slides were incubated with 50 ng/ml DAPI to stain the nuclei. The coverslips were mounted on VECTASHIELD reagent (Vector Laboratories, Burlingame, CA) and fluorescent images were taken using confocal microscopy (Carl Zeiss Meditec, Inc., Thornwood, NY). Cells incubated with 1% BSA alone were served as negative controls.

Nuclear/Membrane Fractionation

Cells were washed, lysed with a hypotonic buffer [10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.9), 1.5 mmol/L MgCl2, 10 mmol/L KCl, 10% glycerol, 0.5 mmol/L PMSF, and 1 mmol/L dithiothreitol (DTT)], and incubated on ice for 15 min. Five µl of 10% NP-40 was added to each lysate. After centrifugation at 13,200 rpm for 10 min, the supernatant was collected as the membrane/cytosolic fraction. The nuclear pellet were lysed with buffer solution containing 20 mmol/L HEPES buffer (pH 7.9), 1.5 mmol/L MgCl2, 400 mmol/L NaCl, 0.2 mmol/L EDTA, 20% glycerol, 0.5 mmol/L PMSF, and 1 mmol/L DTT, and collected by centrifugation at 13,200 rpm for 10 min at 4°C.

Detection of Reactive Oxygen Species (ROS)

Cells were seeded at 4 × 104 cells/ml in 96 well plates. Adhered cells were incubated in the dark with DCFH-DA to 2′,7′-dichlorofluorescein (1 h/37°C; 40 µM, ThermoFisher Sci). Cells were washed and subjected to different drug treatments for 2h. Fluorescence was analyzed in a plate reader (PHERAstar FS, BMG LABTECH) with excitation at 485 nm and emission at 520 nm.

DNA Damage Detection

Tumors from mice that received DMSO or minoxidil were excised and processed for cryosectioning. 12 mm thick sections were stained with DAPI (100 ng/µl) and imaged using a Leica SPE confocal microscope (Leica Microsystems). The number of nuclei exhibiting fragmented versus normal morphology was counted manually in ImageJ software. Data are expressed as a fraction of the normalized total number of nuclei.

Neutral comet assay was performed for detecting DNA double-strand breaks. The images were captured on a fluorescent microscope and quantified by using TriTek CometScore™.

Echocardiogram and ECG Analysis

Cardiac function was assessed using echocardiography (Vevo 2100 with MS400 series transducer, Visualsonics Inc.). Briefly, mice were anesthetized with 1.2%–1.5% isoflurane and placed on a heating pad to maintain body temperature. Parasternal short-axis 2-D echocardiograms and M-mode cine loops were taken at the level of the papillary muscles. The total length of anesthesia was less than 10 min. Evaluation of stored data was performed offline by a sonographer blind to treatment groups. ECG analysis was performed in LabChart8 (ADInstruments)

Immunohistochemistry (IHC)

Tumors isolated from control mice (DMSO) and one mouse treated with minoxidil were fixed with PFA, embedded in paraffin and sectioned with microtome. Antigene retrieval from tissue sections was performed using sodium citrate, blocked in appropriate serum and incubated overnight with γH2AX (Cell Signaling Technology) at 37C and then with secondary biotynilated antibodies for 30 min at RT. Couterstaining with hematoxylin was performed. Tissues were observed with a Zeiss Axioscope 2 and digital images were acquired with Zeiss Axiocam digital camera and Axiovision software.

CONCLUSIONS

Our work adds important insights on the effects of pharmacological activation of K^+ channel for the treatment of cancer. Due to the considerable limited genomic/proteomic data, animal model for different gynecologic cancers and the extended heterogeneous nature of cancers of the female reproductive system we cannot predict at this time what percent of the cancer patients would benefit from using minoxidil. However, in consideration of the limited therapeutic options for ovarian cancers, we propose that repurposing a well-known, readily available and low-cost non-cancer drug as minoxidil might provide a possible rapid transition from bench to clinic and offer an opportunity to address existing unmet patient needs.

AUTHOR'S NOTE

This work is dedicated Estella (Lidia) Cronk, Nicoletta De Canale and Craig Beeson. 'Anything in existence, having somehow come about, is continually interpreted anew, requisitioned anew, transformed and redirected to a new purpose.' (F. Nietzsche).

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB: LU209031, Loyola University Chicago. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by IRB: LU209031, Loyola University Chicago Institutional Animal Care and Use Committee guidelines (Animal Assurance No. D16-00074).

AUTHOR CONTRIBUTIONS

DF-L, AH, VS, GB, and CB have contributed in the design and execution of the experiments in the reported study. AR, VS, ML, JB, RP, and SG have contributed in the conception and

interpretation of the data in the reported study. ML, JB, and SG have contributed in writing the manuscript.

FUNDING

This research was funded by The Cronk Foundation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00577/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | (A) PCR analyses of Kir6.1, Kir6.2, SUR1, SUR2A and SUR2B in the ovarian cancer cells OVCAR-4, OVCAR-8 and SPEC-2. **(B)** Percent of tumor samples from the TCGA database with under/over expression of SUR2 gene. Different cut-offs of expression z-scores are indicated. **(C)** Kaplan–Meier plots of overall survival in patients with ovarian cancer comparing the patients with high (red) and low (black) expression of SUR2 (top vs. bottom tertiles). Probe: Affy ID: 208462_s_at; Datasets: GSE14764, GSE23554, GSE26193, GSE26712, GSE30161, GSE3149, GSE32062, GSE63885, GSE9891, TCGA. **(D)** Representative results showing the effect of minoxidil in the SUR2 negative cell line OVCAR-4. **(E)** Western blot analyses of proliferation markers phosphorylated Wee1, phosphorylated CDK1 and cyclin A in OVCAR-8 treated with or without minoxidil (50mg/ml; 24hr).

SUPPLEMENTARY FIGURE 2 | (A, B) FACS (PI) analysis showing the rescue effect of MitoTEMPO on the lethal effect of minoxidil on OVCAR-8 cells.

REFERENCES

Bao, L., Hadjiolova, K., Coetzee, W. A., and Rindler, M. J. (2011). Endosomal KATP channels as a reservoir after myocardial ischemia: a role for SUR2 subunits. *Am. J. Physiol. Heart Circ. Physiol.* 300 (1), H262–H270. doi: 10.1152/ajpheart.00857.2010

Beech, D. J., and Cheong, A. (2006). Potassium channels at the beginnings of cell proliferation. *J. Physiol.* 570 (Pt 1), 1. doi: 10.1113/jphysiol.2005.101873

Bolduc, C., and Shapiro, J. (2000). Management of androgenetic alopecia. *Am. J. Clin. Dermatol.* 1 (3), 151–158. doi: 10.2165/00128071-200001030-00002

Bull, S. C., and Doig, A. J. (2015). Properties of protein drug target classes. *PloS One* 10 (3), e0117955. doi: 10.1371/journal.pone.0117955

Campbell, J. D., Sansom, M. S., and Ashcroft, F. M. (2003). Potassium channel regulation. *EMBO Rep.* 4 (11), 1038–1042. doi: 10.1038/sj.embor.7400003

Fleishaker, J. C., and Phillips, J. P. (1989). Adinazolam pharmacokinetics and behavioral effects following administration of 20-60 mg oral doses of its mesylate salt in healthy volunteers. *Psychopharmacol. (Berl.)* 99 (1), 34–39. doi: 10.1007/BF00634449

Fukushiro-Lopes, D. F., Hegel, A. D., Rao, V., Wyatt, D., Baker, A., Breuer, E. K., et al. (2018). Preclinical study of a Kv11.1 potassium channel activator as antineoplastic approach for breast cancer. *Oncotarget* 9 (3), 3321–3337. doi: 10.18632/oncotarget.22925

Garlid, K. D., and Halestrap, A. P. (2012). The mitochondrial K(ATP) channel–fact or fiction? *J. Mol. Cell Cardiol.* 52 (3), 578–583. doi: 10.1016/j.yjmcc.2011.12.011

Gentile, S. (2016). hERG1 potassium channel in cancer cells: a tool to reprogram immortality. *Eur. Biophys. J.* 45 (7), 649–655. doi: 10.1007/s00249-016-1169-3

Khanna, N., Malhotra, R. S., Mehta, A. K., Garg, G. R., Halder, S., and Sharma, K. K. (2011). Interaction of morphine and potassium channel openers on experimental models of pain in mice. *Fundam. Clin. Pharmacol.* 25 (4), 479–484. doi: 10.1111/j.1472-8206.2010.00880.x

Kikuchi, S., Fujita, Y., Onodera, M., Fujino, Y., and Inoue, Y. (2016). Prolonged hypotension induced by ingesting a topical minoxidil solution: analysis of minoxidil and its metabolites. *Acute Med. Surg.* 3 (4), 384–387. doi: 10.1002/ams2.196

Landry, L. G., Ali, N., Williams, D. R., Rehm, H. L., and Bonham, V. L. (2018). Lack Of Diversity In Genomic Databases Is A Barrier To Translating Precision Medicine Research Into Practice. *Health Aff. (Millwood)* 37 (5), 780–785. doi: 10.1377/hlthaff.2017.1595

Lansu, K., and Gentile, S. (2013). Potassium channel activation inhibits proliferation of breast cancer cells by activating a senescence program. *Cell Death Dis.* 4, e652. doi: 10.1038/cddis.2013.174

Mannhold, R. (2004). KATP channel openers: structure-activity relationships and therapeutic potential. *Med. Res. Rev.* 24 (2), 213–266. doi: 10.1002/med.10060

Mfuh, A. M., and Larionov, O. V. (2015). Heterocyclic N-Oxides - An Emerging Class of Therapeutic Agents. *Curr. Med. Chem.* 22 (24), 2819–2857. doi: 10.2174/0929867322666150619104007

Miller, R. P., Tadagavadi, R. K., Ramesh, G., and Reeves, W. B. (2010). Mechanisms of Cisplatin nephrotoxicity. *Toxins (Basel)* 2 (11), 2490–2518. doi: 10.3390/toxins2112490

Nagy, A., Lanczky, A., Menyhart, O., and Gyorffy, B. (2018). Validation of miRNA prognostic power in hepatocellular carcinoma using expression data of independent datasets. *Sci. Rep.* 8 (1), 9227. doi: 10.1038/s41598-018-27521-y

Perez-Neut, M., Rao, V. R., Haar, L., Jones, K. W., and Gentile, S. (2015). Current and Potential Antiarrhythmic Drugs Targeting Voltage-Gated Cardiac Ion Channels. *Cardiol. Pharmacol.* 4 (139), 1–7. doi: 10.4172/2329-6607.1000139

Perez-Neut, M., Haar, L., Rao, V., Santha, S., Lansu, K., Rana, B., et al. (2016). Activation of hERG3 channel stimulates autophagy and promotes cellular senescence in melanoma. *Oncotarget* 7 (16), 21991–22004. doi: 10.18632/oncotarget.7831

Pettinger, W. A. (2017). Hypertension's 3 Dilemmas and 3 Solutions: Pharmacology of the Kidney in Hypertension. *J. Cardiovasc. Pharmacol.* 69 (3), 129–139. doi: 10.1097/FJC.0000000000000458

Rao, V. R., Perez-Neut, M., Kaja, S., and Gentile, S. (2015). Voltage-gated ion channels in cancer cell proliferation. *Cancers (Basel)* 7 (2), 849–875. doi: 10.3390/cancers7020813

Reagan-Shaw, S., Nihal, M., and Ahmad, N. (2008). Dose translation from animal to human studies revisited. *FASEB J.* 22 (3), 659–661. doi: 10.1096/fj.07-9574LSF

Rhodes, D. R., Yu, J., Shanker, K., Deshpande, N., Varambally, R., Ghosh, D., et al. (2004). ONCOMINE: a cancer microarray database and integrated data-mining platform. *Neoplasia* 6 (1), 1–6. doi: 10.1016/S1476-5586(04)80047-2

Ries, F., and Klastersky, J. (1986). Nephrotoxicity induced by cancer chemotherapy with special emphasis on cisplatin toxicity. *Am. J. Kidney Dis.* 8 (5), 368–379. doi: 10.1016/S0272-6386(86)80112-3

Rivera, R., and Guerra-Tapia, A. (2008). [Management of androgenetic alopecia in postmenopausal women]. *Actas Dermosifiliogr.* 99 (4), 257–261. doi: 10.1016/S0001-7310(08)74673-8

Salari, S., Ghasemi, M., Fahanik-Babaei, J., Saghiri, R., Sauve, R., and Eliassi, A. (2015). Evidence for a KATP Channel in Rough Endoplasmic Reticulum (rerKATP Channel) of Rat Hepatocytes. *PloS One* 10 (5), e0125798. doi: 10.1371/journal.pone.0125798

Sato, T., Li, Y., Saito, T., and Nakaya, H. (2004). Minoxidil opens mitochondrial K(ATP) channels and confers cardioprotection. *Br. J. Pharmacol.* 141 (2), 360–366. doi: 10.1038/sj.bjp.0705613

Shaul, Y. D., Yuan, B., Thiru, P., Nutter-Upham, A., McCallum, S., Lanzkron, C., et al. (2016). MERAV: a tool for comparing gene expression across human tissues and cell types. *Nucleic Acids Res.* 44 (D1), D560–D566. doi: 10.1093/nar/gkv1337

Shorter, K., Farjo, N. P., Picksley, S. M., and Randall, V. A. (2008). Human hair follicles contain two forms of ATP-sensitive potassium channels, only one of which is sensitive to minoxidil. *FASEB J.* 22 (6), 1725–1736. doi: 10.1096/fj.07-099424

Tomita, M., Kurata, H., Aoki, Y., Tanaka, K., and Kazama, J. J. (2001). Pharmacokinetics of paclitaxel and cisplatin in a hemodialysis patient with recurrent ovarian cancer. *Anticancer Drugs* 12 (5), 485–487. doi: 10.1097/00001813-200106000-00010

Urrego, D., Tomczak, A. P., Zahed, F., Stuhmer, W., and Pardo, L. A. (2014). Potassium channels in cell cycle and cell proliferation. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 369 (1638), 20130094. doi: 10.1098/rstb.2013.0094

Villanueva, J., Infante, J. R., Krepler, C., Reyes-Uribe, P., Samanta, M., Chen, H. Y., et al. (2013). Concurrent MEK2 mutation and BRAF amplification confer resistance to BRAF and MEK inhibitors in melanoma. *Cell Rep.* 4 (6), 1090–1099. doi: 10.1016/j.celrep.2013.08.023

Wang, Z. (2004). Roles of K+ channels in regulating tumour cell proliferation and apoptosis. *Pflugers Arch.* 448 (3), 274–286. doi: 10.1007/s00424-004-1258-5

Zhang, Y., Yuan, F., Cao, X., Zhai, Z., GangHuang,, Du, X., et al. (2014). P2X7 receptor blockade protects against cisplatin-induced nephrotoxicity in mice by decreasing the activities of inflammasome components, oxidative stress and caspase-3. *Toxicol. Appl. Pharmacol.* 281 (1), 1–10. doi: 10.1016/j.taap.2014.09.016

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 funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Copyright © 2020 Fukushiro-Lopes, Hegel, Russo, Senyuk, Liotta, Beeson, Beeson, Burdette, Potkul and Gentile. 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 Pharmacology

ORIGINAL RESEARCH
published: 14 May 2020
doi: 10.3389/fphar.2020.00686

A Novel Anti-Kv10.1 Nanobody Fused to Single-Chain TRAIL Enhances Apoptosis Induction in Cancer Cells

*Franziska Hartung[1], Thomas Krüwel[2], Xiaoyi Shi[1], Klaus Pfizenmaier[3], Roland Kontermann[3], Patrick Chames[4], Frauke Alves[2,5] and Luis A. Pardo[1]**

[1] Oncophysiology Group, Max Planck, Institute of Experimental Medicine, Göttingen, Germany, [2] Institute of Diagnostic and Interventional Radiology, University Medical Center Göttingen, Göttingen, Germany, [3] Institut für Zellbiologie und Immunologie, Universität Stuttgart, Stuttgart, Germany, [4] Aix Marseille Univ, CNRS, Inserm, Institut Paoli-Calmettes, CRCM, Marseille, France, [5] Translational Molecular Imaging Group, Max Planck Institute of Experimental Medicine, Göttingen, Germany

OPEN ACCESS

Edited by:
Lin-Hua Jiang, University of Leeds, United Kingdom

Reviewed by:
Heike Wulff, University of California, Davis, United States
Vladimir Yarov-Yarovoy, University of California, Davis, United States

***Correspondence:**
Luis A. Pardo
pardo@em.mpg.de

Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *13 March 2020*
Accepted: *27 April 2020*
Published: *14 May 2020*

Citation:
Hartung F, Krüwel T, Shi X, Pfizenmaier K, Kontermann R, Chames P, Alves F and Pardo LA (2020) A Novel Anti-Kv10.1 Nanobody Fused to Single-Chain TRAIL Enhances Apoptosis Induction in Cancer Cells. Front. Pharmacol. 11:686. doi: 10.3389/fphar.2020.00686

Antibody-based therapies hold promise for a safe and efficient treatment of cancer. The identification of target tumor cells through a specific antigen enriched on their surface and the subsequent delivery of the therapeutic agent only to those cells requires, besides the efficacy of the therapeutic agent itself, the identification of an antigen enriched on the surface of tumor cells, the generation of high affinity antibodies against that antigen. We have generated single-domain antibodies (nanobodies) against the voltage-gated potassium channel Kv10.1, which outside of the brain is detectable almost exclusively in tumor cells. The nanobody with highest affinity was fused to an improved form of the tumor necrosis factor-related apoptosis inducing ligand TRAIL, to target this cytokine to the surface of tumor cells. The resulting construct, VHH-D9-scTRAIL, shows rapid and strong apoptosis induction in different tumor models in cell culture. The construct combines two sources of specificity, the expression of the antigen restricted to tumor cells and the tumor selectivity of TRAIL. Such specificity combined with the high affinity obtained through nanobodies make the novel agent a promising concept for cancer therapy.

Keywords: targeted therapy, nanobody, TRAIL, Kv10.1, voltage-gated potassium channel, apoptosis

INTRODUCTION

Antibody engineering is an essential process to improve the efficacy of antibody-based therapeutics. Beside the whole monoclonal antibody (mAb) formats with their two antigen-binding arms and Fc-region, which can be modified and gain diverse effector functions, small antibody fragments, like single-chain fragments (scFv), diabodies, or nanobodies are in the focus of innovative therapeutic and diagnostic strategies. Nanobodies, the variable domain of (VHH) heavy-chain antibodies from camelids, play an essential role in this context, because of their particular stability and small size (15 kDa) (Muyldermans, 2013; Chanier and Chames, 2019). The VHH domain consists of four framework regions (FR) and three highly variable loops, the complementarity determining regions (CDR), which form the antigen-binding interface (paratope) and determine the nanobody specificity.

Despite the reduced size of their paratope, nanobodies can bind their target antigen with affinities comparable to those observed in mAbs (Muyldermans et al., 2001).

In this study we describe the design of a nanobody-based affinity protein targeting the tumor-associated antigen Kv10.1, a voltage-gated potassium channel overexpressed in different tumor tissues and many cancer cell lines (Pardo and Stühmer, 2014; Ouadid-Ahidouch et al., 2016) and references therein). We have been interested for many years in the use of voltage-gated potassium channels, and specifically Kv10.1, as therapeutic targets in cancer. Outside of the brain, Kv10.1 is shortly expressed at the end of the cell cycle, where it participates in the finalization of mitosis. Therefore, at any given time, very few non-neuronal cells express the channel on their surface (Sánchez et al., 2016; Urrego et al., 2016). This time-dependent expression pattern is tightly controlled, and therefore Kv10.1 is at the crossroads of many transcription factors and non-coding RNAs (e.g., (Lin et al., 2011; Bai et al., 2013; Wu et al., 2013; Sánchez et al., 2016; Urrego et al., 2016; Horst et al., 2017; Hsu et al., 2017)). The complex regulation is often lost in tumors, and Kv10.1 becomes abundantly expressed in a majority of human cancers (e.g., (Hemmerlein et al., 2006; Ding et al., 2007; Spohr et al., 2007; Ding et al., 2008; Menendez et al., 2012; Lai et al., 2014)), indicating that its function is beneficial for the cancer cell. Inhibition of channel function has been proposed as a therapeutic strategy (Gómez-Varela et al., 2007; Downie et al., 2008; De Guadalupe Chavez-Lopez et al., 2015), and there is evidence that this approach can prolong survival in patients with brain metastases (Martínez et al., 2015). Alternatively, the channel can be used as a marker for the tumor cell due to its scarce expression in normal tissues outside of the brain (Hartung and Pardo, 2016). Based on structural analysis of our already developed mouse-derived scFv antibody (Gómez-Varela et al., 2007) and the newly isolated anti-Kv10.1 nanobodies, we generated a new fusion construct with the apoptosis-inducing ligand TRAIL (Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand) (Wiley et al., 1995).

TRAIL is an attractive candidate for cancer treatment and for the design of antibody-fusion constructs since it has been demonstrated that it is able to selectively induce apoptosis in cancer (Ashkenazi et al., 1999). TRAIL is expressed on the surface of immune cells and binds to five different receptors. Two of them, TRAIL-R1 and TRAIL-R2, induce caspase activation and apoptotic cell death after ligand binding. TRAIL-R3 and TRAIL-R4 are decoy receptors, also expressed on the cell surface but lacking functional intracellular death domains. Membrane-bound TRAIL (on the membrane of immune cells) shows increased ability to induce receptor clustering and more efficient activation of the apoptotic signaling (Muhlenbeck et al., 2000; Von Karstedt et al., 2017), and this can be mimicked by functionalization with agents binding to surface antigens (Wajant et al., 2001). Conversely, soluble TRAIL has also been shown to induce an inflammatory response that can result in higher migration of the tumor cells and thereby tumor spread (Zhou DH, 2013; von Karstedt et al., 2015; Hartwig et al., 2017). Active targeting of TRAIL to cancer cells by fusion with an antibody moiety targeting a tumor-associated antigen enriched in tumor cells results not only in increased accumulation of the fusion proteins at the tumor site, but also mimics membrane-bound presentation of TRAIL, thus allowing higher efficacy and less inflammatory response. We and others have already shown the improved activity of antibody-TRAIL fusion constructs (Stieglmaier et al., 2007; Bremer et al., 2008; Hartung et al., 2011; Hartung and Pardo, 2016).

The homotrimeric structure of active TRAIL can lead to decreased therapeutic efficiency by dissociation into the monomeric subunits. To overcome this drawback, in this study we used the single-chain variant of TRAIL (scTRAIL), a fusion of three single TRAIL fragments *via* short peptide linkers that shows enhanced apoptosis induction (Seifert et al., 2014; Hutt et al., 2018; Siegemund et al., 2018). The properties of nanobodies (small size, high stability and solubility, high affinity (Jovcevska and Muyldermans, 2020) have been already used in combination with TRAIL. Nanobodies against EGFR fused to TRAIL have shown efficacy against tumor cells resistant to both strategies (inhibition of EGFR and activation of TRAIL) when used separately (Zhu et al., 2017).

In this study, we describe a high affinity construct, VHH-D9-scTRAIL, that targets a TRAIL variant with enhanced proapoptotic activity to tumor cells in cell culture models. The construct combines the specificity of Kv10.1 as tumor-associated antigen with the small size and high stability of nanobodies and the efficacy of scTRAIL as a promising candidate to overcome resistance to conventional chemotherapy.

RESULTS

Generation of Anti-Kv10.1 VHH Nanobodies

Anti-Kv10.1 nanobodies were generated by immunization of a llama with a Kv10.1-derived antigen, already successful in generating mouse anti-Kv10.1 mouse mAb (Hemmerlein et al., 2006). The antigen encompasses the E3 segment of the channel, which corresponds to the extracellular linker between S5 and S6 transmembrane segments and is remarkably long in this channel family, and extends to the pore loop. With the aim to induce tetramerization of the target sequences, E3 was fused to the C-terminal tetramerizing coiled-coil of the channel (Jenke et al., 2003). The resulting antibody response is therefore likely to target the extracellular (exposed) domains. The construct contains also thioredoxin (TRX) to increase solubility and stability (Lavallie et al., 1993). **Figure S1** shows a schematic view of the antigen and its conservation among mammalian species. After immunization, the resulting phage display library of $1.3x10^7$ clones was rescued within the helper phage KM13 and enriched through 9 rounds of depletion on immobilized TRX and incubation with different concentrations of the antigen. 186 clones where then screened on the antigen and negatively on TRX, resulting in 30 hits. Of those, ten clones were amplified and induced for nanobody production. Sequencing revealed nine independent clones (**Figure 1**). A detailed look to the primary structure of those binders revealed clustering into two classes,

with pronounced differences in complementarity determining regions (CDR) 2 and 3. Nanobodies VHH-C4 and VHH-D9 shared a remarkably short CDR 3 and therefore had a lower molecular weight compared to all other tested clones (**Figure S2**). CDR1 was relatively conserved in all antibodies, and the framework (FR) regions were conserved throughout all positive binders.

We next estimated the apparent affinities of the VHH antibodies for the recombinant antigen by ELISA (**Figure 2A**). All nanobodies showed affinities in the submicromolar range, but the two clones with shorter CDR3 (C4 and D9) had clearly higher apparent affinities (K_d of 81 nM and 11 nM respectively; **Table 1**, **Figure 2A**). It is worth pointing out that those two antibodies share sequence homology with the mouse monoclonal

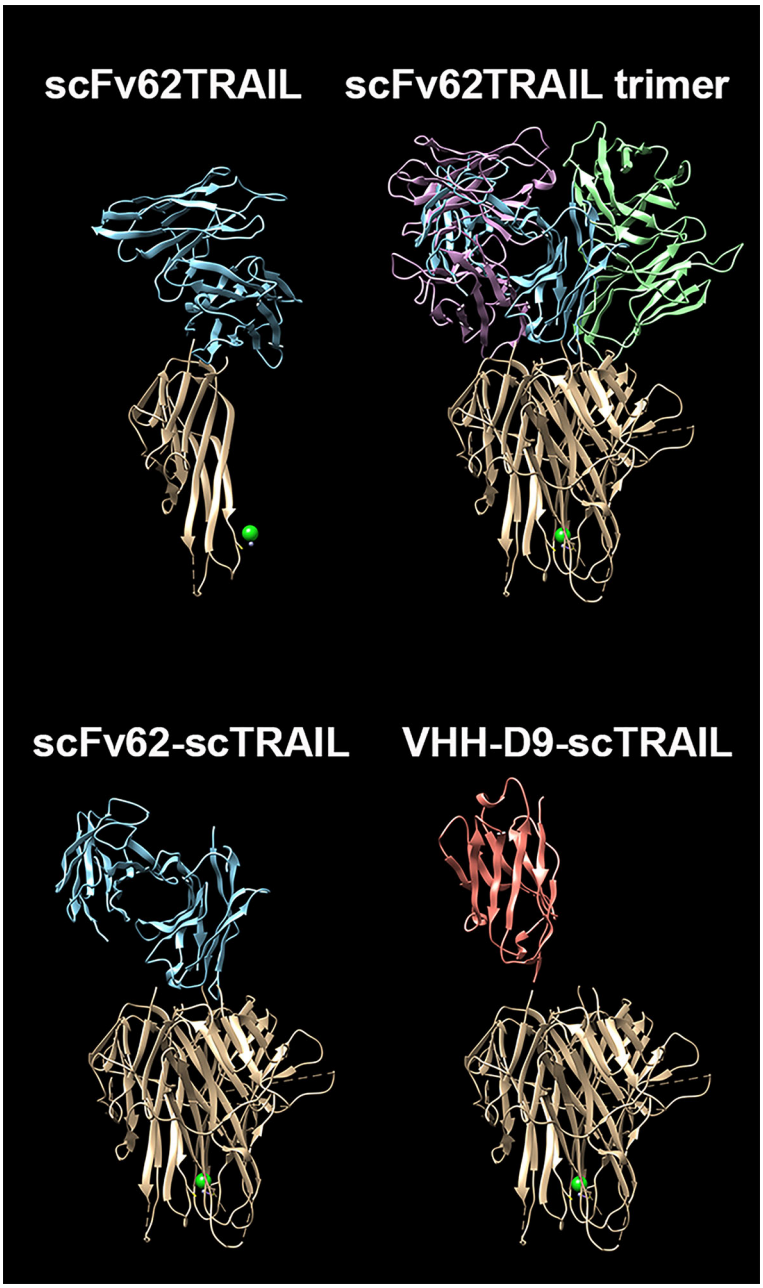


FIGURE 1 | Sequence alignment of the anti-Kv10.1 nanobodies. CDR1, 2, and 3 are underlined. Residues are colored according to chemistry. Note the strong similarity between C4 and D9, especially the conserved CDR2 and the short CR3.

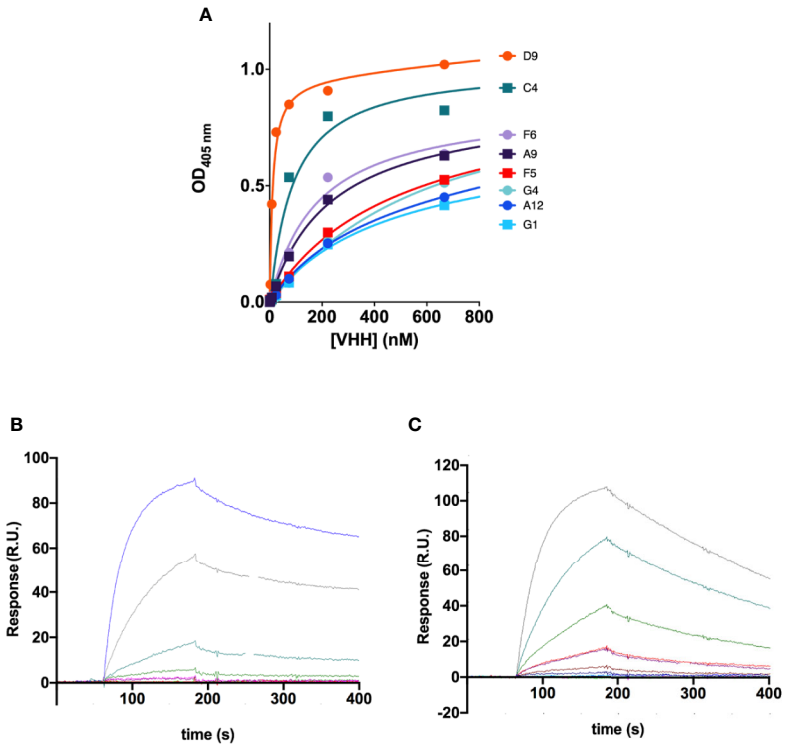


FIGURE 2 | Affinity of the anti-Kv10.1 nanobodies. **(A)** Apparent affinities of the VHH antibodies determined by ELISA against the antigen used for immunization. The values resulting from the fit are listed in **Table 1**. D9 and C4 showed the highest affinity. **(B)** Affinity and kinetics of binding of VHH C4 on h1x immobilized on a surface plasmon resonance (SPR) sensor chip. The concentration used in each sesorgram is listed in the main text. R.U.: resonance units. **(C)** SPR sensorgrams for nanobody D9.

antibody showing highest affinity against this region in our previous studies (mAb62), which was the starting sequence for our single chain antibody scFv62 (Hartung et al., 2011).

The affinity of these two antibodies was also assessed by surface plasmon resonance (SPR) to determine affinity (K_D), association (k_a), and dissociation (k_d) constants. A serial dilution of nanobodies D9 (3.12 nM to 200 nM, **Figure 2B**) and C4 (25 nM to 400 nM, **Figure 2C**) was flown as analyte over the recombinant antigen used for immunization immobilized on the SPR chip. Affinities of 1.8 μM and 78 nM for C4 and D9, respectively, were determined by using a 1:1 Langmuir model resulting in comparable K_D values as previously determined for D9. Interestingly, C4 is characterized by both, a slower association and a slower dissociation compared to D9.

Construction of VHH-D9-scTRAIL Fusions

Given its superior binding properties, we selected the nanobody D9 for subsequent studies. Our model for the design of a targeted therapy against Kv10.1 includes proteins fused to TRAIL, which have shown efficacy *in vitro* and *in vivo*. Since single chain TRAIL (scTRAIL, a tandem repeat of three TRAIL subunits to maintain trimeric structure) is superior in inducing apoptosis to monomeric TRAIL (Siegemund et al., 2018), we decided to use this strategy for the VHH fusion. scTRAIL was also fused to our previously reported scFv62 antibody (Hartung et al., 2011) to compare the behavior of the two types of antibodies. **Figure 3** shows schematically the structural differences of our validated scFv62-TRAIL (Hartung et al., 2011) and the new scTRAIL fusions. The constructs were produced in CHO-K1 cells using the pSecTag system to get the product secreted into the medium.

TABLE 1 | Apparent affinity of the nanobodies determined by ELISA.

Nanobody	A9	A12	C4	D9	F5	F6	G1	G4
K_d (nM)	223	343	87	11	470	177	431	943

VHH-D9-scTRAIL Maintains Affinity and Specificity

We first checked whether fusion of the antibodies to scTRAIL impairs the affinities and/or the specificities of the antibodies. The two scTRAIL fusions, scFv62-scTRAIL and VHH-D9-scTRAIL were analyzed for their binding affinity to the recombinant antigen h1x, which is the same protein used to rise both antibodies. We also tested affinity for a similar protein derived from the closely related channel Kv10.2, that is, the E3 domain of Kv10.2 and its tetramerizing coiled-coil (h2x). As shown in **Figure 4**, the apparent affinity to h1x and h2x was

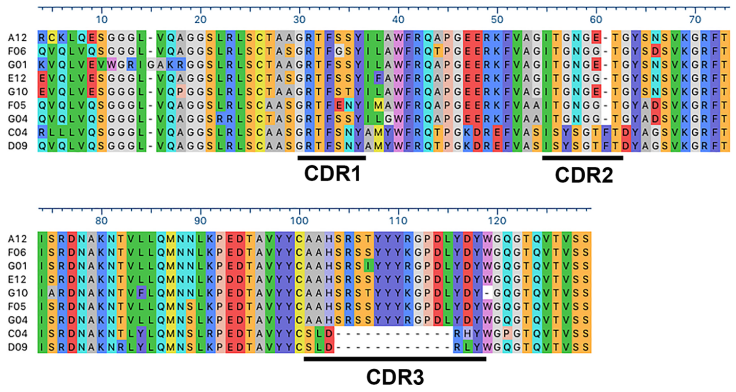


FIGURE 3 | Comparison of the predicted structures of the constructs. The three-dimensional structure of scFv62 as a monomer, its assembly as a functional trimer, and the three-dimensional (3-D) structures of scFv62-scTRAIL and VHH-D9-scTRAIL are depicted with the different moieties in different colors (blue, green and magenta for scFv62, red for VHH and light brown for TRAIL). The TRAIL trimer structure corresponds to PDB ID 1dg6 (Hymowitz et al., 2000). The structures of scFv62 and VHH-D9 have been modelled using AbodyBuilder (Leem et al., 2016). The PDB files can be found in Supplementary Information.

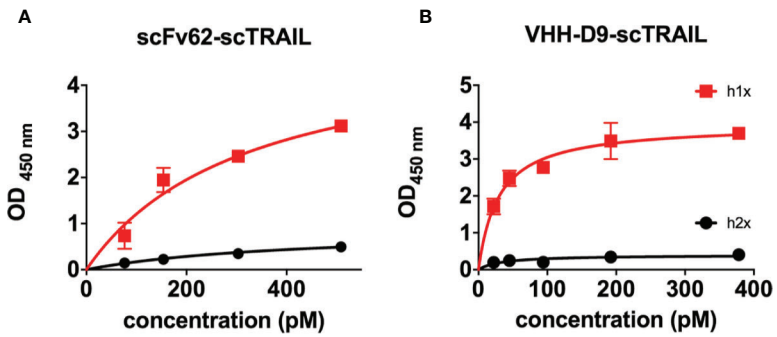


FIGURE 4 | Apparent affinity and selectivity of scFv62-scTRAIL and VHH-D9-scTRAIL. h1x and h2x antigens were immobilized on ELISA plates and incubated with different concentrations of the relevant fusion proteins. Binding of the fusion was measured by detection of TRAIL. Results on scFv62-scTRAIL are shown in **(A)**, and those of VHH-D9-scTRAIL in panel **(B)**. Black line and symbols correspond to h2x, which produced weak signals in both cases. Red color indicates data obtained with h1x. Error bars indicate standard deviation.

assessed by ELISA. Plates were coated with the antigen (h1x or h2x) and a commercial TRAIL ELISA kit was used for detection. It is unclear how accurate the absolute values for apparent affinity are because of the stable trimeric structure of TRAIL in the construct, but the relative values should be informative as to how scFv and VHH compare to each other. The apparent affinities of scFv62-scTRAIL and VHH-D9-scTRAIL constructs measured in these experiments were 280 pM and 29 pM, respectively. The large difference observed between the apparent affinity of VHH-D9-scTRAIL and of VHH-D9 alone may be explained by the different methods used to detect the nanobody alone (His-Tag, one epitope per antibody molecule) and the fusion construct (TRAIL, which is trivalent). VHH-D9-scTRAIL showed a 10-fold higher apparent affinity to the antigen than scFv62-scTRAIL. Therefore, both constructs retain affinity for h1x. Both also maintain specificity, because none of the constructs showed binding to h2x (**Figure 4**, black symbols).

VHH-D9-scTRAIL Induces Apoptosis in Kv10.1 Positive Tumor Cells

In the next step, we studied the efficacy of the VHH-D9-scTRAIL construct in inducing apoptosis in Kv10.1-expressing human prostate cancer cells (DU-145) in conventional cell culture. To analyze the process of apoptosis induction we used the live-cell imaging system IncuCyte and a caspase assay to quantify apoptosis induction over time. Activation of caspase 3/7 is reported as an increase in fluorescence (**Figure 5A**).

Due to its low toxicity in the time window tested, cycloheximide (CHX) was used subsequently as sensitizer for all *in vitro* experiments. **Figure 5A** shows the development of the apoptotic signal in DU-145 cells over time after treatment with different concentrations of the two scTRAIL fusion constructs over time. Comparison of the apoptotic signals in response to scFv62-scTRAIL and VHH-D9-scTRAIL shows more efficient and faster apoptosis induction by VHH-D9-scTRAIL starting

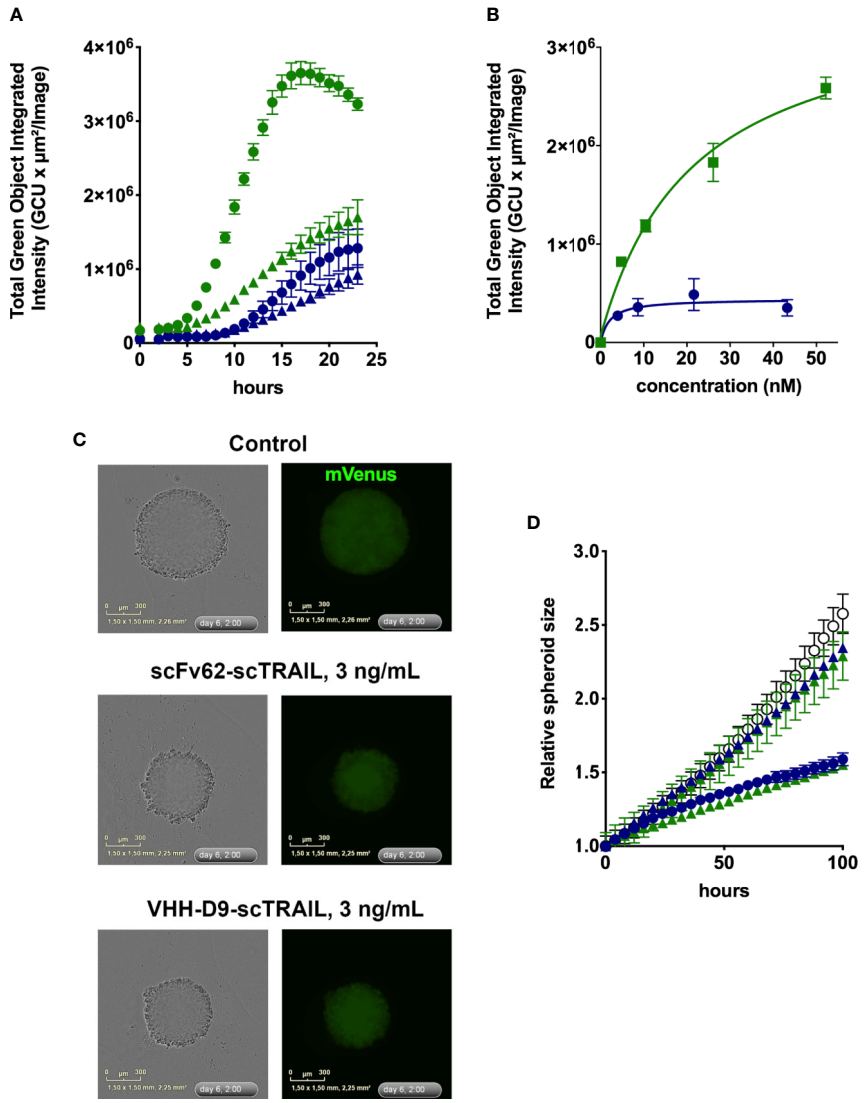


FIGURE 5 | Apoptosis induction by scFv62-scTRAIL and VHH-D9-scTRAIL on DU-145 cells. **(A)** Cells seeded in 96 well plates were treated with 370 pg/ml (triangles) or 3.7 ng/ml (circles) of scFv62-scTRAIL (corresponding to 4.3 pM and 43 pM, blue) or VHH-D9-scTRAIL (corresponding to 5.2 pM and 52 pM, green), and apoptosis was measured over time using a green fluorescent caspase reporter. All conditions induced apoptosis, but especially VHH-D9-scTRAIL produced a large fraction of apoptotic cells as early as 8 h after the start of treatment. **(B)** Dose-response of the apoptosis induction by scFv62-scTRAIL (blue) and VHH-D9-scTRAIL (green). **(C)** Representative images of spheroids produced by fluorescent (mVenus) DU-145 cells and submitted to the indicated treatments. **(D)** Relative size of the spheroids treated with the fusion constructs at 300 pg/ml and 3 ng/ml (corresponding to 4.2 and 42 pM for VHH-D9-scTRAIL and 3.5 and 35 pM for scFV62-scTRAIL). The values in response to lower doses of the constructs did not differ from the control (open black circles), while higher doses reduced spheroid size for both scFv62-scTRAIL (blue) and VHH-D9-scTRAIL (green). All data presented as means ± SD.

after 4 h of treatment. Analysis of the apoptotic signals at 12 h after treatment and different concentrations of both scTRAIL constructs illustrates the clear difference in the intensity of apoptosis induction between them, three times more in the case of VHH-D9-scTRAIL, although its EC50 (~20 nM) was higher than that of scFv62-scTRAIL (~2 nM; **Figure 5B**).

In terms of response to therapy, it is generally accepted that three-dimensional (3-D) cultures are more similar to the real tumors than 2-D cultures. Because a relevant difference between the two constructs is size, likely affecting the ability to penetrate the tumor, we set out to test efficacy of the constructs on the growth and viability of tumor spheroids. We used a DU-145-derived cell line that expresses constitutively the fluorescence reporter protein mVenus (Hartung et al., 2011). We measured over time the change in size of the spheroids by determining the surface occupied by green fluorescence by live cell imaging in the IncuCyte system (**Figure 5C**). Both constructs inhibited the growth of the spheroids with similar efficacy (**Figure 5D**);

the spheroids grew one-half of the controls during the time of the experiment in the presence of higher doses of scTRAIL constructs (3 ng/ml).

The lack of a difference in efficacy between both fusion constructs was surprising, because VHH-D9-scTRAIL was more potent than scFv62-scTRAIL in 2-D cultures, and it is also expected to have improved tumor penetration and higher affinity, and thereby a stronger effect in spheroids. We hypothesized that this could be due to the fast growth of DU-145 spheroids. Therefore, we performed similar experiments using the pancreatic cancer cell line Capan-1, which on one hand shows slower growth, and on the other hand is strongly influenced by co-culture with stromal cells.

In standard 2-D culture, both VHH-D9-scTRAIL and scFv62-scTRAIL effectively induced apoptosis in the presence of CHX in Capan-1 cells. As expected, VHH-D9-scTRAIL had a more intense effect, corresponding to its higher affinity to Kv10.1 (**Figure 6A**). When grown as single culture, spheroids of Capan-1 cells showed remarkable apoptosis in the presence of both scTRAIL constructs, both in quantitative terms (**Figure 6B**) and morphologically (**Figure 6C** and **Video S1**). VHH-D9-scTRAIL was again remarkably more efficacious than scFv62-scTRAIL inducing apoptosis in this model. The effect was even more evident when the spheroids were formed by a mixture of Capan-1 and stellate cells (RLT-PSCs (Jesnowski et al., 2005); 4,000 cells of each type at the time of seeding). In order to distinguish between tumor and stellate cells, RLT-PSC were stably transfected with the red fluorescent reporter mCherry protein. Live imaging of the spheroids (**Figure 6D**, **Video S1**) revealed that the stellate cells were not affected by the presence of the constructs, but the apoptosis induction was fast and intense in the tumor cells, measured as green fluorescence originated by cleavage of caspase 3/7 substrate, with apoptosis already observed 12 h after the start of treatment (**Figure 6E**). We used the surface occupied by red fluorescence from the RLT-PSC to normalize for the size of the spheroid. The lower dose of VHH-D9-scTRAIL used (300 pg/mL) was similarly efficacious as the higher dose of scFv62-scTRAIL (3 ng/ml).

DISCUSSION

In this work, we have combined the specificity for tumor cells and accessibility of Kv10.1 with the advantages of nanobodies and of scTRAIL to generate a fusion construct with enhanced apoptosis inducing activity targeting different tumor cells.

The use of scTRAIL, besides enhanced efficacy, leads to size reduction, since the construct contains just only one antibody unit instead of three; however, it also requires higher affinity antibodies. scTRAIL has been shown to bind with affinity of approximately 1 nM to the TRAIL-R2 receptor; therefore, to take advantage of binding to the tumor antigen, the affinity of the antibody needs to be higher (Hutt et al., 2018). Therefore, to improve antibody affinity, we generated a series of single domain antibodies, which can achieve very high binding affinity combined with smaller size and better stability (Xenaki et al., 2017; Chanier and Chames, 2019). Two of the isolated VHHs showed high apparent binding affinity, in one case (D9) in the low nM range. Interestingly, the two nanobodies with highest apparent affinity show a remarkably short CDR3, with just six residues. Comparative analysis of 90 different nanobody sequences identifies a mean CDR3 length of 15 residues (Mitchell and Colwell, 2018), which also holds true for most of the VHHs identified in our screening.

We show that the two constructs carrying scTRAIL, scFv62-scTRAIL, and VHH-D9-scTRAIL retain high affinity for the antigen and are selective between Kv10.1 and its closest relative, Kv10.2 (Ludwig et al., 2000), indicating that the furoin to the cargo did not alter the binding activity of the antibody moiety. The apparent affinity of VHH-D9-scTRAIL, in the pM range as determined by ELISA, was much higher than that of the VHH before fusion (nM). This could be due to the different approaches used to detect binding. For the VHH alone, ELISA detection used the His-tag from the vector for phage display. The tag was removed to generate the fusion construct, and therefore we needed to use an anti-TRAIL ELISA for detection of VHH-D9-scTRAIL. The presence of three TRAIL subunits per molecule of VHH could be responsible for the apparently higher affinity.

The efficacy of apoptosis induction of the new constructs was tested *in vitro* in DU-145 cells, a human prostate cancer cell line that had served already as a model for scFv62-TRAIL, a construct using monomeric TRAIL (Hartung et al., 2011; Hartung and Pardo, 2016). In this study, DU-145 cells also showed to be highly sensitive to the scTRAIL-based fusion, with and EC50 in the low nanomolar range. The effect of VHH-D9-scTRAIL required higher dose despite its higher affinity for the antigen (EC50 in the tens of nM), but the onset of the effect was faster and apoptosis induction was more intense than that of scFv62-scTRAIL. In spheroid experiments, however, both constructs were similarly efficacious in terms of growth inhibition. We did observe very intense apoptosis in DU-145 spheroids treated with both constructs, but the setup of the experiment makes quantification difficult. DU-145 spheroids are very compact, and do not change morphology upon treatment. Experiments using, e.g., two-photon microscopy will be needed for a more accurate quantification of apoptosis induction. Another factor that could influence the outcome is the fast growth of DU-145 cells (doubling time of approximately 29 h), which doubled the diameter of the spheroid in approximately 72 h.

In the human pancreatic cancer derived Capan-1, characterized by a much slower growth (doubling time of approximately 60–80 h), both fusion proteins induced intense apoptosis in standard 2D culture, with VHH-D9-scTRAIL being more potent than scFv62-scTRAIL. Tumor spheroids formed by Capan-1 cells alone are less compact than DU-145 spheroids, but compact spheroids were formed in the presence of RLT-PSC stellate stromal cells. Under both conditions, single culture or co-culture, both fusion constructs induced strong apoptosis also in this cell model. Clear apoptosis was detectable as early as 12 h after the start of treatment when using 3 ng/ml of the constructs, earlier in the case of VHH-D9-scTRAIL. The lower

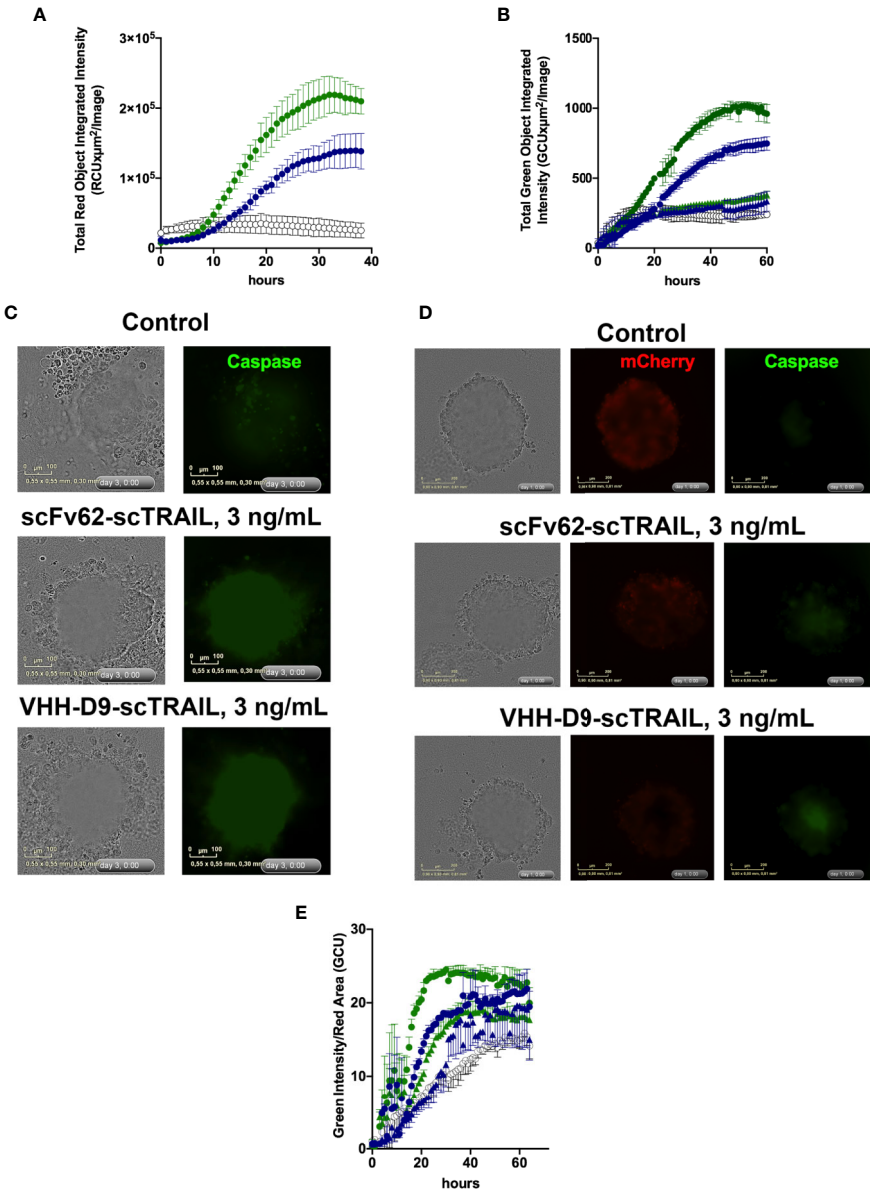


FIGURE 6 | Apoptosis induction by scFv62-scTRAIL and VHH-D9-scTRAIL on Capan-1 cells. **(A)** Cells seeded in 96 well plates were treated with 3 ng/ml of scFv62-scTRAIL (35 pM, blue) or VHH-D9-scTRAIL (42 pM, green), and apoptosis was measured over time using a green fluorescent caspase reporter. VHH-D9-scTRAIL produced a larger fraction of apoptotic cells. **(B)** Apoptosis induction in Capan-1 spheroids of 0.3 ng/ml (triangles) or 3 ng/ml (circles) VHH-D9-scTRAIL (green) or scFv62-scTRAIL. The effects were similar to those observed in two-dimensional (2-D) culture, albeit delayed in time. **(C)** Representative images of spheroids produced by Capan-1 cells and treated as indicated. Green fluorescence indicates caspase 3/7 activation. **(D)** Representative images of spheroids produced by Capan-1 cells co-cultured with stellate cells and treated as indicated. Red fluorescence identifies stellate cells (mCherry), while green corresponds to caspase activation. **(E)** Apoptosis induction in Capan-1 spheroids cocultured with RLT-PSCs expressing mCherry relative to the size of the compact spheroid. Apoptosis was detected as green fluorescence, and the size of the compact spheroid was inferred from the area of red fluorescence from stellate cells. There was fast and intense induction of apoptosis when 3 ng/ml (circles) VHH-D9-scTRAIL (green) or scFv62-scTRAIL were added to the medium. 0.3 ng/ml (triangles) showed little apoptosis induction. All data presented as means ± SD.

concentration used, 300 pg/ml, was also efficacious in the case of VHH-D9-scTRAIL, while the effect was less clear for scFv62-scTRAIL.

In summary, we generated in this study novel nanobody-based antibody-scTRAIL fusion constructs, whose binding unit shows high affinity for Kv10.1 and that are able to efficiently induce apoptosis of cancer cells. VHH-D9-scTRAIL was consistently faster and more potent than the scFv version.

Further investigations and *in vivo* studies are necessary to characterize other properties of VHH-D9-scTRAIL. The small size and remarkable stability of nanobodies as compared to other antibody forms should be advantageous in the therapeutic

setting, but pharmacokinetic studies including tissue penetration, biodistribution, stability, and blood clearance will be required to confirm this assumption. Importantly, also for the potential use of the nanobody for diagnostic applications, it remains to be demonstrated that the increased efficacy of VHH-D9-scTRAIL is due to its high affinity for the antigen. It is still possible that the nanobody fusion induces a conformational change in scTRAIL that results in more efficient apoptosis induction, a possibility that merits careful consideration. The antigenic region is virtually identical in all Kv10.1 mammalian orthologs. This level of conservation will be important to predict potential undesired effects in preclinical models.

MATERIAL AND METHODS

Immunization and Phage Display

An adult male llama (*Lama glama*) was immunized subcutaneously at days 1, 20, 41, and 65 with 100 μg of the h1x antigen (E3-S5-pore segment fused to the C-terminal coiled coil domain of Kv10.1 and to thioredoxin). A phage display library was built as previously described (Behar et al., 2009). Briefly, the genes of VHH were amplified by RT-PCR using total RNA extracted from purified peripheral blood mononuclear cell and cloned into a phagemid vector to generate library of 1.3×10^7 transformants. The library was created by the Nanobody platform of CRCM (Cancer Research Center of Marseille).

Phage display was performed as described (Even-Desrumeaux and Chames, 2012). For depletion against TRX, selection was performed using immobilized TRX or h1x on magnetic M450 Epoxy beads (Life Technologies). Both beads and phages were blocked with 20% BSA in PBS for 1 h at 4°C, and then incubated with the immobilized TRX (2 h, 4°C). The supernatant of the TRX slurry was then incubated on immobilized h1X for selection. The beads were washed 9 times with PBS plus 0.1% Tween 20 and twice with PBS and treated with 1 mg/ml trypsin for 30 min to elute the phages. 186 colonies of bacteria (*E. coli* $TG1_{TR}$) infected with the output phages (3 sterile controls) were picked, grown overnight and transferred to round bottom 96 well plates and induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight. The culture supernatants were used for screening by the enzyme linked immunosorbent assay (ELISA).

scFv62-scTRAIL and VHH-D9-scTRAIL Constructs

Design and construction of the mouse-derived scFv has been described earlier (Hartung et al., 2011). The nanobodies against Kv10.1. The scTRAIL sequence was cloned together with scFv62, or VHH D9 into the multiple cloning site of the pSecTag2A plasmid. The fusion protein was expressed without the tags encoded in the pSecTag2A plasmid, which were eliminated by mutagenesis. The pSecTag2A protein expression vector with the corresponding insets was transfected into CHO-K1 cells. The plasmid carries the murine *kappa* light-chain leader peptide upstream of the multiple cloning site, and therefore directs the produced fusion protein through the ER and Golgi, resulting in excretion to the culture supernatant. Single clones were isolated from the transfected CHO-K1 cells and selected for those that showed the highest levels of secreted Ab-scTRAIL into the medium. Transfected cells were selected with Zeocin (3μg/ml in culture medium). For overexpression, the cells were cultured in a protein- and serum-free CHO medium [Panserin C6000 (PAN Biotech)] for five days at 30°C. The presence of the desired construct in the culture supernatant was confirmed by Western blot. No signal from other proteins was detected after electrophoresis and Comassie blue staining, and the supernatants were used without further purification. The product concentration was determined using the Human Quantikine TRAIL ELISA kit (R&D Systems) according to the manufacturer protocol against immobilized h1x. The expression yields ranged from 200-300 μg/L of culture.

ELISA

96-well plates were coated overnight with 500 ng of h1x, h2x or TRX in 100 μl TBS per well in a wet chamber at room temperature. To block unspecific binding, wells were blocked with 2% (VHH ELISA) or 3% BSA (TRAIL ELISA) in TBS or PBS for 1 h. The VHHs (*E. coli* culture supernatants) or Ab-scTRAIL constructs were incubated at different concentration in 200 μl TBS for 2 h with shaking, and washed three times. VHH were detected by mouse anti-His antibody (Millipore) and a secondary anti-mouse, peroxidase conjugate (GE Healthcare). TRAIL signals were detected using anti-TRAIL conjugate and the detection buffer (R&D Systems, Quantikine TRAIL ELISA kit) according to the manufacturer's protocol.

Cell Culture

DU-145 and Capan-1, were purchased from ATCC, and CHO-K1 from DSMZ. Capan-1 cells were cultured in RPMI 1640 with 10%FCS; DU-145 in DMEM with 10% FCS and CHO-K1 cells in Ham's F-12 medium with 10% FCS at 37°C in humidified 5% CO_2 atmosphere. All media were purchased from Gibco Thermo Fisher Scientific. Transfection of Ab-scTRAIL in CHO-K1 cells was done with Lipofectamine 3000 (Thermo Fisher Scientific) as recommended by the manufacturer.

Spheroids were cultured in round bottom ultra-low attachment 96-well plates (Corning) at a density of 5,000 cells/well or 4,000 Capan-1 and 4,000 RLT-PSC cells per well in 2% Matrigel (Corning) and centrifuged at 1000x*g* for 10 min. Spheroid formation was monitored continuously in the IncuCyte system and treatments were added once spheroids were formed.

Apoptosis Measurement

Cells or spheroids seeded in 96-well plates were monitored using the IncuCyte live-cell imaging system Essen Biosciences). Cells and spheroids were treated with the Ab-scTRAIL constructs in the presence of 8.9 μM cycloheximide (CHX) and Caspase 3/7 reagent (Essen Biosciences, red or green as required; 1:1,000 as recommended by the manufacturer). Caspase 3/7 reagent substrate crosses the cell membrane and its cleavage by activated caspase-3/7 results in the release of a DNA dye and fluorescent staining of nuclear DNA.

Statistical Analysis

Data were analyzed using GraphPad Prism v.8 and are represented as mean ± SD. At least two biological replicates were performed for each analysis. Monolayer cell culture experiments were performed in triplicates, with two images per well and time point. Spheroid experiments were imaged also in triplicate wells, with a single image per well (covering the whole spheroid) at each time point.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

FH, TK, PC, FA, and LP conceived and designed the study. KP and RK provided materials. FH, TK, and XS performed experiments, FH, TK, XS, PC, FA, and LP participated in data analysis and interpretation. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This project has received funding from the Max-Planck Society and from the European Union through Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 813834-PHIONIC-H2020-MSCA-ITN-2018, and through the FP7 NMP "Large" Project NAMDIATREAM (contract no.246479). Open access publication costs funded by the Max-Planck-Society.

ACKNOWLEDGMENTS

We thank Kerstin Dümke, Bärbel Heidrich, and Víctor Díaz for expert technical assistance. T RLT-PSC stellate cells were a gift from Dr. Ralf Jeneofsky, University of Heidelberg. The mCherry-expressing cell line was constructed by Dr. Farrah Zahed.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00686/full#supplementary-material

SUPPLEMENTARY VIDEO S1-6 | Coordinate files for scFv62 and VHHD9 models in PDB format.

SUPPLEMENTARY FIGURE 1 | Overview of the antigen used. **(A)** Aminoacid sequence of h1x. The Thioredoxin tag, E3 and C-terminal assembly domains are indicated by bars. **(B)** Alignment of the relevant sequences of h1x and Kv10.1 from several mammalian species. E3 is conserved among all species shown, and only a single residue in bovine and rat Kv10.1, and two in mouse, differ from the human sequence in the C-terminal assembly domain. The numbering corresponds to Homo sapiens Kv10.1, isoform 1 (UniProt O95259-2). UniProt IDs are: Pan troglodytes, H2Q129; Bos taurus, O18965; Rattus norvegicus, Q63472; Mus musculus, Q60603.

SUPPLEMENTARY FIGURE 2 | Western blot of anti-Kv10.1 lead candidates. Purity and size of selected anti-Kv10.1 nanobodies lead candidates were validated by Western blot. Nanobodies were expressed in E. coli TG1TR. Three μg of the purified nanobodies were separated by SDS-PAGE. The blotted proteins were revealed with a HRP conjugated anti-His antibody. The nanobodies were expressed as monomers with a molecular weight ranging from 14 - 17 kDa (arrow). The higher molecular weight bands (bracket) correspond to the fusion between the nanobody and the p3 protein from the phage M13, as well as some degradation products from this p3.

REFERENCES

Ashkenazi, A., Pai, R. C., Fong, S., Leung, S., Lawrence, D. A., Marsters, S. A., et al. (1999). Safety and antitumor activity of recombinant soluble Apo2 ligand. *J. Clin. Invest*. 104, 155–162. doi: 10.1172/JCI6926

Bai, Y., Liao, H., Liu, T., Zeng, X., Xiao, F., Luo, L., et al. (2013). MiR-296-3p regulates cell growth and multi-drug resistance of human glioblastoma by targeting ether-a-go-go (EAG1). *Eur. J. Cancer* 49, 710–724. doi: 10.1016/j.ejca.2012.08.020

Behar, G., Chames, P., Teulon, I., Cornillon, A., Alshoukr, F., Roquet, F., et al. (2009). Llama single-domain antibodies directed against nonconventional epitopes of tumor-associated carcinoembryonic antigen absent from nonspecific cross-reacting antigen. *FEBS J.* 276, 3881–3893. doi: 10.1111/j.1742-4658.2009.07101.x

Bremer, E., Bruyn, M., Samplonius, D. F., Bijma, T., Ten Cate, B., Leij, L. F. M. H., et al. (2008). Targeted delivery of a designed sTRAIL mutant results in superior apoptotic activity towards EGFR-positive tumor cells. *Int. J. Mol. Med.* 86, 909–924. doi: 10.1007/s00109-008-0348-9

Chanier, T., and Chames, P. (2019). Nanobody Engineering: Toward Next Generation Immunotherapies and Immunoimaging of Cancer. *Antibodies (Basel)* 8. doi: 10.3390/antib8010013

De Guadalupe Chavez-Lopez, M., Perez-Carreon, J. I., Zuniga-Garcia, V., Diaz-Chavez, J., Herrera, L. A., Caro-Sanchez, C. H., et al. (2015). Astemizole-based anticancer therapy for hepatocellular carcinoma (HCC), and Eag1 channels as potential early-stage markers of HCC. *Tumour Biol.* 36, 6149–6158. doi: 10.1007/s13277-015-3299-0

Ding, X. W., Luo, H. S., Jin, X., Yan, J. J., and Ai, Y. W. (2007). Aberrant expression of Eag1 potassium channels in gastric cancer patients and cell lines. *Med. Oncol.* 24, 345–350. doi: 10.1007/s12032-007-0015-y

Ding, X. W., Wang, X. G., Luo, H. S., Tan, S. Y., Gao, S., Luo, B., et al. (2008). Expression and prognostic roles of Eag1 in resected esophageal squamous cell carcinomas. *Dig. Dis. Sci.* 53, 2039–2044. doi: 10.1007/s10620-007-0116-7

Downie, B. R., Sánchez, A., Knötgen, H., Contreras-Jurado, C., Gymnopoulos, M., Weber, C., et al. (2008). Eag1 expression interferes with hypoxia homeostasis and induces angiogenesis in tumors. *J. Biol. Chem.* 283, 36234–36240. doi: 10.1074/jbc.M801830200

Even-Desrumeaux, K., and Chames, P. (2012). Phage display and selections on cells. *Methods Mol. Biol.* 907, 225–235. doi: 10.1007/978-1-61779-974-7_12

Gómez-Varela, D., Zwick-Wallasch, E., Knötgen, H., Sánchez, A., Hettmann, T., Ossipov, D., et al. (2007). Monoclonal antibody blockade of the human Eag1 potassium channel function exerts antitumor activity. *Cancer Res.* 67, 7343–7349. doi: 10.1158/0008-5472.CAN-07-0107

Hartwig, T., Montinaro, A., Von Karstedt, S., Sevko, A., Surinova, S., Chakravarthy, A., et al (2017). The TRAIL-Induced Cancer Secretome Promotes a Tumor-Supportive Immune Microenvironment via CCR2. *Molecular Cell* 65, 730–742 e735.doi: 10.1016/j.molcel.2017.01.021

Hartung, F., and Pardo, L. A. (2016). Guiding TRAIL to cancer cells through Kv10.1 potassium channel overcomes resistance to doxorubicin. *Eur. Biophys. J.* 45, 709–719. doi: 10.1007/s00249-016-1149-7

Hartung, F., Stühmer, W., and Pardo, L. A. (2011). Tumor cell-selective apoptosis induction through targeting of KV10.1 via bifunctional TRAIL antibody. *Mol. Cancer* 10, 109. doi: 10.1186/1476-4598-10-109

Hemmerlein, B., Weseloh, R. M., De Queiroz, F. M., Knötgen, H., Sánchez, A., Rubio, M. E., et al. (2006). Overexpression of Eag1 potassium channels in clinical tumours. *Mol. Cancer* 5, 41. doi: 10.1186/1476-4598-5-41

Horst, C. H., Titze-De-Almeida, R., and Titze-De-Almeida, S. S. (2017). The involvement of Eag1 potassium channels and miR-34a in rotenone-induced death of dopaminergic SH-SY5Y cells. *Mol. Med. Rep.* 15, 1479–1488. doi: 10.3892/mmr.2017.6191

Hsu, P.-H., Ma, Y.-T., Fang, Y.-C., Huang, J.-J., Gan, Y.-L., Chang, P.-T., et al. (2017). Cullin 7 mediates proteasomal and lysosomal degradations of rat Eag1 potassium channels. *Sci. Rep.* 7, 40825. doi: 10.1038/srep40825

Hutt, M., Fellermeier-Kopf, S., Seifert, O., Schmitt, L. C., Pfizenmaier, K., and Kontermann, R. E. (2018). Targeting scFv-Fc-scTRAIL fusion proteins to tumor cells. *Oncotarget* 9, 11322–11335. doi: 10.18632/oncotarget.24379

Hymowitz, S. G., O'connell, M. P., Ultsch, M. H., Hurst, A., Totpal, K., Ashkenazi, A., et al. (2000). A Unique Zinc-Binding Site Revealed by a High-Resolution X-ray Structure of Homotrimeric Apo2L/TRAIL. *Biochemistry* 39, 633–640. doi: 10.1021/bi992242l

Jenke, M., Sanchez, A., Monje, F., Stuhmer, W., Weseloh, R. M., and Pardo, L. A. (2003). C-terminal domains implicated in the functional surface expression of potassium channels. *EMBO J.* 22, 395–403. doi: 10.1093/emboj/cdg035

Jesnowski, R., Furst, D., Ringel, J., Chen, Y., Schrodel, A., Kleeff, J., et al. (2005). Immortalization of pancreatic stellate cells as an in vitro model of pancreatic fibrosis: deactivation is induced by matrigel and N-acetylcysteine. *Lab. Invest.* 85, 1276–1291. doi: 10.1038/labinvest.3700329

Jovcevska, I., and Muyldermans, S. (2020). The Therapeutic Potential of Nanobodies. *BioDrugs* 34, 11–26. doi: 10.1007/s40259-019-00392-z

Lai, Q., Wang, T., Guo, Q., Zhang, Y., Wang, Y., Yuan, L., et al. (2014). Positive correlation between the expression of hEag1 and HIF-1alpha in breast cancers: an observational study. *BMJ Open* 4, e005049. doi: 10.1136/bmjopen-2014-005049

Lavallie, E. R., Diblasio Ea Fau - Kovacic, S., Kovacic S Fau - Grant, K. L., Grant Kl Fau - Schendel, P. F., Schendel Pf Fau - Mccoy, J. M., and Mccoy, J. M. (1993). A thioredoxin gene fusion expression system that circumvents inclusion body formation in the E. coli cytoplasm. *Biotechnol. (NY)* 11, 1987–1193. doi: 10.1038/nbt0293-187

Leem, J., Dunbar, J., Georges, G., Shi, J., and Deane, C. M. (2016). ABodyBuilder: Automated antibody structure prediction with data-driven accuracy estimation. *MAbs* 8, 1259–1268. doi: 10.1080/19420862.2016.1205773

Lin, H., Li, Z., Chen, C., Luo, X., Xiao, J., Dong, D., et al. (2011). Transcriptional and post-transcriptional mechanisms for oncogenic overexpression of ether à go-go K^+ channel. *PloS One* 6, e20362. doi: 10.1371/annotation/45b3e6bc-1065-4357-b215-465176dcc269

Ludwig, J., Weseloh, R., Karschin, C., Liu, Q., Netzer, R., Engeland, B., et al. (2000). Cloning and functional expression of rat eag2, a new member of the ether-à-go-go family of potassium channels and comparison of its distribution with that of eag1. *Mol. Cell Neurosci.* 16, 59–70. doi: 10.1006/mcne.2000.0851

Martínez, R., Stühmer, W., Martin, S., Schell, J., Reichmann, A., Rohde, V., et al. (2015). Analysis of the expression of Kv10.1 potassium channel in patients with brain metastases and glioblastoma multiforme: impact on survival. *BMC Cancer* 15, 1–9. doi: 10.1186/s12885-015-1848-y

Menendez, S. T., Villaronga, M. A., Rodrigo, J. P., Alvarez-Teijeiro, S., Garcia-Carracedo, D., Urdinguio, R. G., et al. (2012). Frequent aberrant expression of the human ether à go-go (hEAG1) potassium channel in head and neck cancer: pathobiological mechanisms and clinical implications. *J. Mol. Med. (Berl)* 90, 1173–1184. doi: 10.1007/s00109-012-0893-0

Mitchell, L. S., and Colwell, L. J. (2018). Comparative analysis of nanobody sequence and structure data. *Proteins* 86, 697–706. doi: 10.1002/prot.25497

Muhlenbeck, F., Schneider, P., Bodmer, J. L., Schwenzer, R., Hauser, A., Schubert, G., et al. (2000). The tumor necrosis factor-related apoptosis-inducing ligand receptors TRAIL-R1 and TRAIL-R2 have distinct cross-linking requirements for initiation of apoptosis and are non-redundant in JNK activation. *J. Biol. Chem.* 275, 32208–32213. doi: 10.1074/jbc.M000482200

Muyldermans, S. (2013). Nanobodies: natural single-domain antibodies. *Annu. Rev. Biochem.* 82, 775–797. doi: 10.1146/annurev-biochem-063011-092449

Muyldermans, S., Cambillau, C., and Wyns, L. (2001). Recognition of antigens by single-domain antibody fragments: the superfluous luxury of paired domains. *Trends Biochem. Sci.* 26, 230–235. doi: 10.1016/s0968-0004(01)01790-x

Ouadid-Ahidouch, H., Ahidouch, A., and Pardo, L. A. (2016). TKv10.1 K+ channel: from physiology to cancer. *Pflugers Archiv-European. J. Physiology* 468, 751–762. doi: 10.1007/s00424-015-1784-3

Pardo, L. A., and Stühmer, W. (2014). The roles of K+ channels in cancer. *Nat. Rev. Cancer* 14, 39–48. doi: 10.1038/nrc3635

Sánchez, A., Urrego, D., and Pardo, L. A. (2016). Cyclic expression of the voltage-gated potassium channel KV10.1 promotes disassembly of the primary cilium. *EMBO Rep.* 17, 708–723. doi: 10.15252/embr.201541082

Seifert, O., Plappert, A., Fellermeier, S., Siegemund, M., Pfizenmaier, K., and Kontermann, R. E. (2014). Tetravalent antibody-scTRAIL fusion proteins with improved properties. *Mol. Cancer Ther.* 13, 101–111. doi: 10.1158/1535-7163.MCT-13-0396

Siegemund, M., Schneider, F., Hutt, M., Seifert, O., Muller, I., Kulms, D., et al. (2018). IgG-single-chain TRAIL fusion proteins for tumour therapy. *Sci. Rep.* 8, 7808. doi: 10.1038/s41598-018-24450-8

Spohr, H., Radzun, H. J., Stuhmer, W., Pardo, L., and Hemmerlein, B. (2007). Expression of Eag1 potassium channels in renal cell carcinomas. *Pathol. Res. Pract.* 203, 311–312.

Stieglmaier, J., Bremer, E., Kellner, C., Liebig, T. M., Cate, B., Peipp, M., et al. (2007). Selective induction of apoptosis in leukemic B-lymphoid cells by a CD19-specific TRAIL fusion protein. *Cancer Immunol. Immun.* 57, 233–246. doi: 10.1007/s00262-007-0370-8

Urrego, D., Movsisyan, N., Ufartes, R., and Pardo, L. A. (2016). Periodic expression of Kv10.1 driven by pRb/E2F1 contributes to G2/M progression of cancer and non-transformed cells. *Cell Cycle* 15, 799–811. doi: 10.1080/15384101.2016.1138187

Von karstedt, S., Conti, A., Nobis, M., Montinaro, A., Hartwig, T., Lemke, J., et al (2015). Cancer Cell-Autonomous TRAIL-R Signaling Promotes KRAS-Driven Cancer Progression, Invasion, and Metastasis. *Cancer Cell* 27, 561–573. doi: 10.1016/j.ccell.2015.02.014

Von Karstedt, S., Montinaro, A., and Walczak, H. (2017). Exploring the TRAILs less travelled: TRAIL in cancer biology and therapy. *Nat. Rev. Cancer* 17, 352–366. doi: 10.1038/nrc.2017.28

Wajant, H., Moosmayer, D., Wuest, T., Bartke, T., Gerlach, E., Schonherr, U., et al. (2001). Differential activation of TRAIL-R1 and -2 by soluble and membrane TRAIL allows selective surface antigen-directed activation of TRAIL-R2 by a soluble TRAIL derivative. *Oncogene* 20, 4101–4106. doi: 10.1038/sj.onc.1204558

Wiley, S. R., Schooley, K., Smolak, P. J., Din, W. S., Huang, C.-P., Nicholl, J. K., et al. (1995). Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* 3, 673–682. doi: 10.1016/1074-7613(95)90057-8

Wu, X., Zhong, D., Gao, Q., Zhai, W., Ding, Z., and Wu, J. (2013). MicroRNA-34a inhibits human osteosarcoma proliferation by downregulating ether a go-go 1 expression. *Int. J. Med. Sci.* 10, 676–682. doi: 10.7150/ijms.5528

Xenaki, K. T., Oliveira, S., and Van Bergen En Henegouwen, P. M. P. (2017). Antibody or Antibody Fragments: Implications for Molecular Imaging and Targeted Therapy of Solid Tumors. *Front. Immunol.* 8, 1287. doi: 10.3389/fimmu.2017.01287

Zhou Dh, Y. L., Roder, C., Kalthoff, H., and Trauzold, A. (2013). TRAIL-induced expression of uPA and IL-8 strongly enhanced by overexpression of TRAF2 and Bcl-xL in pancreatic ductal adenocarcinoma cells. *Hepatobiliary Pancreat. Dis. Int.* 12, 94–98. doi: 10.1016/s1499-3872(13)60012-0

Zhu, Y., Bassoff, N., Reinshagen, C., Bhere, D., Nowicki, M. O., Lawler, S. E., et al.(2017). Bi-specific molecule against EGFR and death receptors simultaneously targets proliferation and death pathways in tumors. *Sci. Rep.* 7, 2602. doi: 10.1038/s41598-017-02483-9

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 Hartung, Krüwel, Shi, Pfizenmaier, Kontermann, Chames, Alves and Pardo. 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 Pharmacology

REVIEW
published: 04 June 2020
doi: 10.3389/fphar.2020.00793

P2X7 in Cancer: From Molecular Mechanisms to Therapeutics

Romain Lara[1], Elena Adinolfi[2], Catherine A. Harwood[3], Mike Philpott[4], Julian A. Barden[5], Francesco Di Virgilio[6] and Shaun McNulty[1]*

[1] Biosceptre (UK) Limited, Cambridge, United Kingdom, [2] Department of Medical Sciences, University of Ferrara, Ferrara, Italy, [3] Centre for Cell Biology and Cutaneous Research, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, London, United Kingdom, [4] Centre for Cutaneous Research, Blizard Institute, Bart's & The London School of Medicine and Dentistry, Queen Mary University of London, London, United Kingdom, [5] Biosceptre (Australia) Pty Ltd., Sydney, NSW, Australia, [6] Department of Morphology, Surgery and Experimental Medicine, Section of Pathology, Oncology and Experimental Biology, University of Ferrara, Ferrara, Italy

P2X7 is a transmembrane receptor expressed in multiple cell types including neurons, dendritic cells, macrophages, monocytes, B and T cells where it can drive a wide range of physiological responses from pain transduction to immune response. Upon activation by its main ligand, extracellular ATP, P2X7 can form a nonselective channel for cations to enter the cell. Prolonged activation of P2X7, *via* high levels of extracellular ATP over an extended time period can lead to the formation of a macropore, leading to depolarization of the plasma membrane and ultimately to cell death. Thus, dependent on its activation state, P2X7 can either drive cell survival and proliferation, or induce cell death. In cancer, P2X7 has been shown to have a broad range of functions, including playing key roles in the development and spread of tumor cells. It is therefore unsurprising that P2X7 has been reported to be upregulated in several malignancies. Critically, ATP is present at high extracellular concentrations in the tumor microenvironment (TME) compared to levels observed in normal tissues. These high levels of ATP should present a survival challenge for cancer cells, potentially leading to constitutive receptor activation, prolonged macropore formation and ultimately to cell death. Therefore, to deliver the proven advantages for P2X7 in driving tumor survival and metastatic potential, the P2X7 macropore must be tightly controlled while retaining other functions. Studies have shown that commonly expressed P2X7 splice variants, distinct SNPs and post-translational receptor modifications can impair the capacity of P2X7 to open the macropore. These receptor modifications and potentially others may ultimately protect cancer cells from the negative consequences associated with constitutive activation of P2X7. Significantly, the effects of both P2X7 agonists and antagonists in preclinical tumor models of cancer demonstrate the potential for agents modifying P2X7 function, to provide innovative cancer therapies. This review summarizes recent advances in understanding of the structure and functions of P2X7 and how these impact P2X7 roles in cancer progression. We also review potential therapeutic approaches directed against P2X7.

Keywords: P2X7, cancer, tumor progression, tumor microenvironment, therapeutics

OPEN ACCESS

Edited by:
Stefan Feske,
New York University, United States

Reviewed by:
Friedrich Haag,
University Medical Center Hamburg-Eppendorf, Germany
Stephen Christopher Jameson,
University of Minnesota Twin Cities, United States
Wolfgang Junger,
Harvard Medical School, United States

****Correspondence:***
Romain Lara
romain.lara@biosceptre.com

Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *12 March 2020*
Accepted: *13 May 2020*
Published: *04 June 2020*

Citation:
Lara R, Adinolfi E, Harwood CA, Philpott M, Barden JA, Di Virgilio F and McNulty S (2020) P2X7 in Cancer: From Molecular Mechanisms to Therapeutics. Front. Pharmacol. 11:793. doi: 10.3389/fphar.2020.00793

INTRODUCTION

Extracellular ATP is a danger associated molecular pattern (DAMP), which can act through the P2 receptor family including ionotropic P2X ion channel receptors and the G protein-coupled P2Y receptor family (Abbracchio and Burnstock, 1994; Brake et al., 1994; Valera et al., 1994; North, 2002; Burnstock, 2014). The P2X receptor family is formed by seven members able to assemble into homo- and hetero-trimers at the cell membrane (North, 2002; Burnstock, 2006). P2X family members are distinguished by their relative affinities to ATP, within the nanomolar range for P2X1 and P2X3, the low micromolar range for P2X2, P2X4, and P2X5 and the hundreds of micromolar range for P2X7 and also their rate of channel desensitization, milliseconds for P2X1 and P2X3, seconds for P2X2, P2X4, and P2X5 and no observable desensitization for P2X7, (Koshimizu et al., 1999; North and Surprenant, 2000; North, 2002; Burnstock, 2006; Jarvis and Khakh, 2009; Yan et al., 2010; Coddou et al., 2011).

P2X7 is characterized by its biphasic response to ATP stimulation. Upon activation with ATP for a relatively short period (2s or less), P2X7 opens a nonselective cation channel facilitating Na^+ and Ca^{2+} influx and K^+ efflux while prolonged ATP stimulation (4s or above) leads to the formation of a macropore permeable to molecules of <900 Da. However, recent reports have shown that ATP activated P2X7 receptors can open a $NMDG^+$-permeable macropore within milliseconds and without progressive dilatation (Harkat et al., 2017; Pippel et al., 2017). Formation of this large molecular weight-permeable pore has been associated with the release of ATP in the microenvironment (Pellegatti et al., 2005; Adinolfi et al., 2010). Extracellular ATP helps to sustain macropore opening and is likely to drive several physiological and pathological mechanisms including chronic pain (Sorge et al., 2012), proliferation and activation of microglia (Bianco et al., 2006; Monif et al., 2009) and the death of enteric neurons during colitis (Gulbransen et al., 2012). In healthy tissues, P2X7 is expressed mainly in the central nervous system (CNS), peripheral nervous system (PNS), and in immune cells, which supports its emergence as a target for chronic pain and chronic inflammatory diseases (De Marchi et al., 2016). P2X7 is also overexpressed in a number of cancer types, potentially driving tumor development and survival (Adinolfi et al., 2002; Adinolfi et al., 2009; Adinolfi et al., 2012; Barden et al., 2014; Amoroso et al., 2015; Barden et al., 2016; Gilbert et al., 2019).

As a consequence of its ion channel activity, P2X7 is involved in driving several functions known to be important during tumor initiation and progression (Di Virgilio et al., 2018a). Yet, analysis of sequencing repositories from large tumor panels, including deep sequencing initiatives, does not highlight the preferential selection of mutation or copy number variation in the P2RX7 gene by tumor cells (Lawrence et al., 2014). This suggests that other receptor control mechanisms might modulate the critical role P2X7 plays during cancer progression. Extracellular ATP is present at low levels in healthy tissues but increases dramatically in response to tissue damage, inflammation, hypoxia, ischaemia and in the tumor microenvironment (TME). This increase in extracellular ATP has been attributed to cell death, cell stress, vesicular release, activation of pannexin and connexin, and to ATP-binding cassette (ABC) transporters (Pellegatti et al., 2008; Eltzschig et al., 2012; Galluzzi et al., 2017). In the TME, extracellular ATP is hydrolyzed into AMP by the ectonucleotidase CD39, AMP is then hydrolyzed into adenosine by the CD73 ectonucleotidase (Allard et al., 2017). While extracellular ATP acts as a DAMP to trigger proinflammatory immune responses, adenosine was found to be a potent immunosuppressor (Di Virgilio et al., 2018a). In this context, P2X7 collaboration with purinergic related proteins such as CD39 and Pannexin 1, was found to play an important role in the regulation of the antitumor immune response (de Andrade Mello et al., 2017).

The high extracellular ATP concentration found in the TME is sufficient to open the P2X7 macropore that could in turn lead to membrane depolarization and ultimately to cell death. To support tumor progression, cancer cells need to inhibit P2X7 macropore activity while retaining other receptor signaling functions. Several studies have described molecular mechanisms that lead to loss or attenuation of macropore function (See **Table 1**). These include the report of a misfolded form of P2X7 termed nonfunctional P2X7 (nfP2X7) that is characterized by the exposure of an extracellular epitope named E200 (Gidley-Baird and Barden, 2002; Barden et al., 2014; Gilbert et al., 2019). In this review we seek to describe how the interplay between P2X7 functions drive tumor progression and immune escape. We first present the topological features, functions and processes activated downstream of the P2X7 receptor before discussing how the interplay between these features may explain the paradoxical role of P2X7 in the tumor and the immune compartment. We then discuss the expression and role of P2X7 across multiple cancer types before reviewing the therapeutic approaches taken to target P2X7 to date.

Topology of the P2X7 Receptor

The human *P2RX7* gene is located on chromosome 12 and encodes 13 exons that translate into a 595 amino acid protein. The location of *P2RX7* (12q24.31) is adjacent to the *P2RX4* gene, which is only 20Mbp downstream in the same reading direction (Buell et al., 1998a). Both genes are believed to be derived from successive gene duplications (Dubyak, 2007; Hou and Cao, 2016). Indeed, a recent report suggests that P2X7 was probably formed in lower vertebrates through the fusion of a P2X4-like gene with a Zn-coordinating cysteine-based domain (ZCD) coding exon (Rump et al., 2020). While heteromerisation of P2X7 and P2X4 *in vivo* is still controversial, both genes are found to be widely coexpressed (Guo et al., 2007; Kaczmarek-Hajek et al., 2012) and colocalize to act in concert in the regulation of the same physio-pathological functions (Kopp et al., 2019). Thirteen P2X7 splice variants have been identified to date (Benzaquen et al., 2019). While the resolution of the structure of human P2X7 has not yet been achieved, due to its propensity to aggregate, the partial structure of human P2X3 (Mansoor et al., 2016), zebra fish P2X4 (Kawate et al., 2009; Hattori and

TABLE 1 | Channel modifications leading to the loss or gain of P2X7 macropore function.

Designation	Type of modification	Outcome	Reference
Modification leading to Loss of P2X7 Macropore function			
V76A	SNP	Partial loss of macropore function	(Stokes et al., 2010; Oyanguren-Desez et al., 2011)
R117W	SNP	Partial loss of channel and macropore function	(Roger et al., 2010; Wiley et al., 2011; Jiang et al., 2013)
G150R	SNP	Loss of macropore function	(Stokes et al., 2010)
E186K	SNP	Loss of channel and macropore function	(Roger et al., 2010; Wiley et al., 2011; Jiang et al., 2013)
N187D	SNP	Possible loss of function	(Chong et al., 2010b)
L191P	SNP	Partial loss of channel and macropore function	(Roger et al., 2010; Wiley et al., 2011; Jiang et al., 2013)
R276H	SNP	Loss of macropore function	(Stokes et al., 2010)
R307Q	SNP	Loss of macropore function	(Gu et al., 2004; Gartland et al., 2012; Jorgensen et al., 2012; Gu et al., 2015)
A348T	SNP	Mild increase of channel function in human P2X7 and mild decrease in channel function in rat P2X7	(Cabrini et al., 2005; Bradley et al., 2011)
T357S	SNP	Partial loss of channel and macropore function	(Cabrini et al., 2005; Shemon et al., 2006)
Q460R	SNP	Partial loss of channel and macropore function	(Cabrini et al., 2005; Stokes et al., 2010)
E496A	SNP	Loss of channel and macropore function	(Gu et al., 2001; Boldt et al., 2003; Cabrini et al., 2005; Roger et al., 2010; Sun et al., 2010; Gidlof et al., 2012; Wesselius et al., 2012; Jiang et al., 2013)
I568N	SNP	Loss of channel and pore function due to impaired P2X7 trafficking to the plasma membrane	(Wiley et al., 2003)
P2X7 variant B	Splice variant	Loss of macropore function (P2X7 variant A and B coexpression leads to gain of macropore function)	(Cheewatrakoolpong et al., 2005; Adinolfi et al., 2010)
P2X7 variant C	Splice variant	Assumed to have lost macropore function	(Cheewatrakoolpong et al., 2005; Benzaquen et al., 2019)
P2X7 variant D	Splice variant	Assumed to have lost macropore function	(Cheewatrakoolpong et al., 2005; Benzaquen et al., 2019)
P2X7 variant E	Splice variant	Assumed to have lost macropore function	(Cheewatrakoolpong et al., 2005; Benzaquen et al., 2019)
P2X7 variant F	Splice variant	Assumed to have lost macropore function	(Cheewatrakoolpong et al., 2005; Benzaquen et al., 2019)
P2X7 variant G	Splice variants	Loss of macropore function	(Cheewatrakoolpong et al., 2005; Benzaquen et al., 2019)
P2X7 variant H	Splice variants	Loss of macropore function	(Cheewatrakoolpong et al., 2005)
P2X7 variant J	Splice variant	Loss of macropore function (P2X7 variant J act as a dominant negative when coexpressed with P2X7 variant A leading to loss macropore function)	(Feng et al., 2006)
N187A	Impaired N-glycosylation	Loss of macropore function	(Lenertz et al., 2010)
R578Q	Impaired N-glycosylation	Loss of macropore function	(Wickert et al., 2013)
R277 or Y298	Proteolytic cleavage	Loss of macropore function following MMP-2 cleavage of P2X7 extracellular domain	(Young et al., 2018)
C-terminal tail	Binding partner	NMMHC-IIA	(Guo et al., 2007; Gu et al., 2009)
TM domains	Cholesterol binding	Loss of macropore function	(Robinson et al., 2014; Karasawa et al., 2017)
C362S and C363S	Prevent cholesterol inhibition rescue	Loss of macropore function	(Karasawa et al., 2017)
Modification leading to Gain of P2X7 Macropore function			
H155Y	SNP	Gain of macropore function	(Cabrini et al., 2005; Stokes et al., 2010; Oyanguren-Desez et al., 2011; Jiang et al., 2013)
A166G	SNP	Gain of macropore function	(Jiang et al., 2013)
H270R	SNP	Gain of macropore function	(Stokes et al., 2010)
R125	ADP-ribosylation of mouse P2X7	Gating of mouse P2X7 macropore	(Adriouch et al., 2008)
R206K, R276K, R277K (mouse)	Mutation	Gain of macropore function in mouse P2X7	(Adriouch et al., 2008)
TM domain	phosphatidylglycerol and sphingomyelin binding	Gain of macropore function	(Karasawa et al., 2017)

Gouaux, 2012; Kasuya et al., 2017), chicken P2X7 (Kasuya et al., 2017), panda P2X7 (Karasawa and Kawate, 2016; Karasawa et al., 2017), and more recently the full-length rat P2X7 (McCarthy et al., 2019) have been resolved. These have begun to reveal the molecular mechanism of ATP channel gating and the topology of the P2X7 trimer at the cell membrane. The P2X7 receptor is divided into five main structural domains (**Figure 1**).

N-Terminal Cytoplasmic Tail

A short N-terminal cytoplasmic tail of 25 amino acids (aa), which is anchored in the membrane *via* the palmitoylation of a cysteine residue at position 4 to form a cytoplasmic cap involved in the sensitisation of the channel to its agonist through key residues such as T15 and Q17 (Yan et al., 2010; McCarthy et al., 2019; Liang et al., 2019).

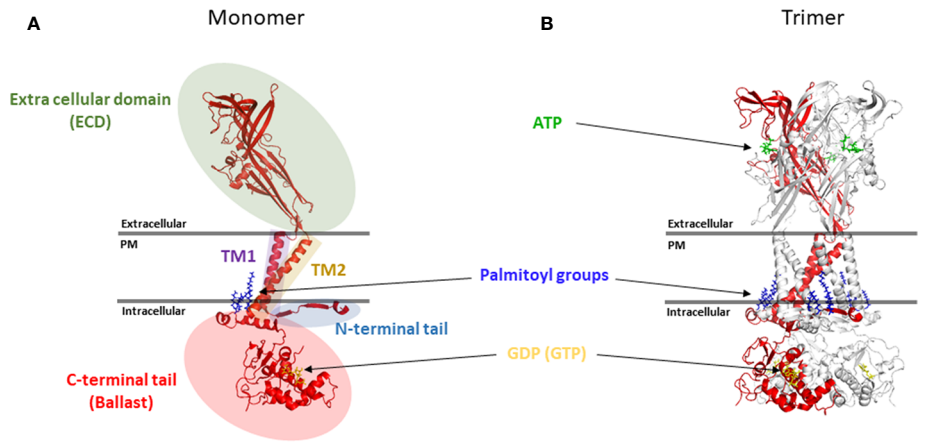


FIGURE 1 | Topology of the P2X7 receptor. **(A)** Five main structural domains are present within each P2X7 monomer **(B)** Positioning of P2X7 monomer in the trimer. Rendering were generated from the rat P2X7 structure (PDB file 6U9W) (McCarthy et al., 2019) and positioned together with ATP, palmitoyl groups and GDP (GTP) molecules in relation to the plasma membrane (PM). Rendering were performed using PyMOL (https://pymol.org/).

First Transmembrane Domain (TM1) and Extracellular Domain

The N-terminal cytoplasmic tail is followed by a first transmembrane domain named TM1 (aa 26 to 46) and a large extracellular domain of 282 aa (aa 47 to 329), which contains an inter-subunit ATP binding pocket (Hansen et al., 1997; Hattori and Gouaux, 2012; Karasawa et al., 2017; McCarthy et al., 2019). The extracellular domain also includes 5 disulfide bonds between cysteine residues 119–168, 129–152, 135–162, 216–226, and 260–269, which play a critical role in maintaining the conformation of the extracellular domain. These disulfide bridges are homologous across the other members of the P2X receptors which suggest that they form highly conserved protein folds (Hansen et al., 1997). Several post-translational modifications have been identified in the P2X7 extracellular domain which can regulate receptor functions; these include ADP-ribosylation of R125 on murine P2X7 that is involved in the gating of the murine P2X7 receptor (Schwarz et al., 2012) and N-linked glycosylation of 5 asparagine residues (D187, D202, D213, D241, and D284) with D187 N-linked glycosylation playing a key role in the regulation of P2X7 signaling through the MAP kinase pathway (Lenertz et al., 2010).

Second Transmembrane Domain (TM2)

The extracellular region is followed by a second transmembrane domain named TM2 (aa 330 to 349), which contains several key pore-lining residues. These include S342, which sits at the narrowest part of the channel to regulate its gating (Pippel et al., 2017; McCarthy et al., 2019). Phosphorylation of the adjacent Y343 also regulates channel gating which further emphasizes the importance of those two residues during channel gating (Kim et al., 2001; Liang et al., 2019).

C-terminal Cytoplasmic Tail

The second transmembrane domain is flanked by a long C-terminal cytoplasmic tail of 245 aa (aa 350 to 595), which acts as a ballast and is involved in the modulation of macropore opening (Surprenant et al., 1996; Cheewatrakoolpong et al., 2005; Costa-Junior et al., 2011; McCarthy et al., 2019). The beginning of the C-terminal tail contains a cysteine rich region that is palmitoylated on at least five residues (C362, C363, C374, C377, and S360) and plays an important role in trafficking of the receptor to the cell membrane and in preventing receptor desensitization (Gonnord et al., 2009; McCarthy et al., 2019). These multiple palmitoylated residues act as a hinge allowing each C-terminal tail to create two zinc binding sites and a guanosine diphosphate or triphosphate (GDP/GTP) binding site. From a functional perspective, the C-terminal tail has been shown to be required for the opening of the macropore (Surprenant et al., 1996; Adinolfi et al., 2010). Recent reports have also shown that pore opening requires movement of the TM2 helix which is directly linked to the globular ballast folded underneath (Pippel et al., 2017; McCarthy et al., 2019). This suggests that a significant movement of the ballast is needed to allow for the macropore to form (See **Figures 1** and **2**). Given the presence of GDP/GTP binding sites in the P2X7 C-terminal tail, it is tempting to hypothesize that such a structural rearrangement may involve an exchange between GDP and GTP. This type of mechanism has already been extensively characterized for GTPase enzymes, which promote the hydrolysis of GTP to GDP to trigger protein interaction and signaling cascades (Bos et al., 2007). This switch could either be mediated by P2X7 itself or through accessory proteins such as GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). However, further work is needed to confirm this hypothesis.

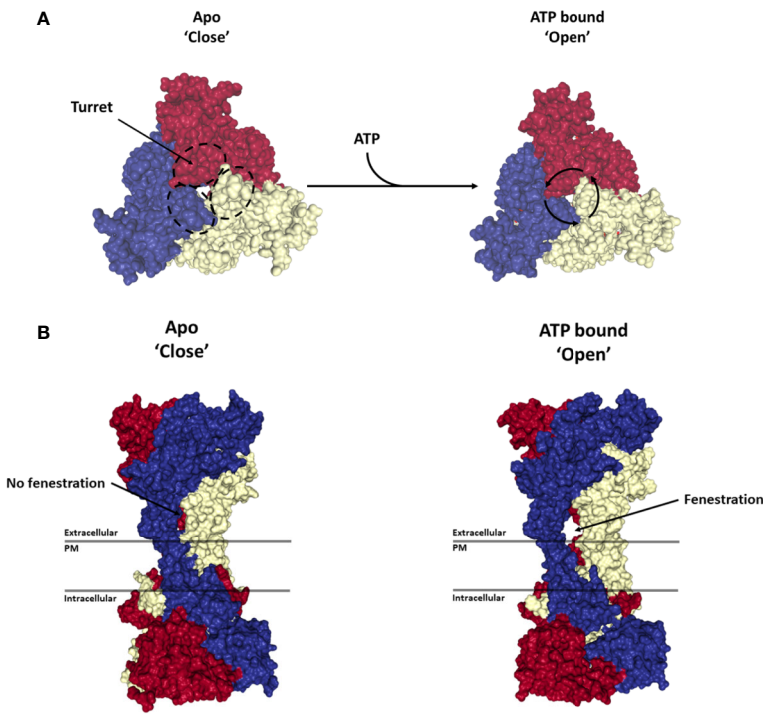


FIGURE 2 | Activation of P2X7 receptor. Structures show the top **(A)** and side view **(B)** of the P2X7 trimer in the Apo-closed state (PDB file: 6U9V) and ATP bound open state (PDB file: 6U9W). Each monomer is represented in a different color (blue, red or yellow). ATP binding to the trimer leads to a rotation of each monomer around its respective turret leading to the opening of a side fenestration and an ion channel through the plasma membrane (PM). Renderings were generated from the rat P2X7 structure (McCarthy et al., 2019) using PyMOL (https://pymol.org/).

P2X7 POSSESSES ION CHANNEL FUNCTIONALITY

P2X7 is distinguished from other P2X family members by its relative insensitivity to its natural agonist ATP (EC_{50} above 80 −100 μM) (Surprenant et al., 1996). ATP binding to the inter-subunit pocket leads to a rotation of each subunit around its turret and a widening of the lower body of the P2X7 trimer. This conformational rearrangement is associated with the opening of a lateral fenestration in the lower body of the trimer that in turn allows influx of Ca^{2+} and Na^{+} ions into and the efflux of K^{+} ions from the cell (**Figures 2A, B**) (Karasawa and Kawate, 2016; McCarthy et al., 2019). Several well characterized small molecule inhibitors have been reported to inhibit this mechanism through binding to an allosteric pocket located in between the turret of each subunit, thereby preventing the closure of the turret necessary for the opening of the channel (**Figures 2A, B**) (Karasawa and Kawate, 2016). Observations made using rat P2X7 suggest that the C-terminal cytoplasmic tail does not significantly regulate receptor current facilitation, desensitization, affinity for ATP or its receptor ion selectivity (McCarthy et al., 2019).

Structural analysis of P2X family members together with experimental characterization and mathematical modeling have provided insights into the transition states of the P2X7 receptor following ATP binding. While occupation of all three ATP binding sites on the trimer leads to a symmetric conformational rearrangement and to channel gating, the binding of a single ATP molecule leads to an asymmetric rearrangement of the trimer leaving the fenestration closed and decreasing the affinity of the second and third ATP binding sites. Upon binding of a second ATP molecule, a low conductance is observed with a further decrease in the affinity of the third ATP binding site (Yan et al., 2010). These observations explain why P2X7 is relatively insensitive to ATP compared to other P2X family members. The functional implication is that P2X7 ion channel activation is only achieved when the level of ATP is significantly elevated in the extracellular compartment. Such conditions can be found in the TME, during inflammatory responses and in chronic inflammatory diseases. In these settings, P2X7 mediated calcium signaling was shown to trigger intracellular signaling and changes in gene expression that are critical for cancer cell survival and proliferation (Di Virgilio, 2015; Di Virgilio et al., 2017; Di Virgilio et al., 2018a). Indeed, overexpression of full length P2X7 was shown to promote tumor growth (Jelassi et al., 2011; Adinolfi et al., 2012a), while P2X7 blockade was shown to inhibit neoplastic growth in several experimental models (Adinolfi et al., 2012a; Adinolfi et al., 2015a; Amoroso et al., 2015; Amoroso et al., 2016; Giannuzzo et al., 2016; De Marchi et al., 2019; Zhang et al., 2019a).

TABLE 2 | Studies confirming the presence of P2X7.

Cancer type	Studies confirming the presence of P2X7
Prostate	(Slater et al., 2004a; Slater et al., 2005; Maianski et al., 2007; Adinolfi et al., 2009; Barden et al., 2009; Ravenna et al., 2009; Adinolfi et al., 2010; Barden et al., 2014; Ghalali et al., 2014; Qiu et al., 2014; Solini et al., 2015; Barden et al., 2016; Gilbert et al., 2019)
Lung	(Barden et al., 2009; Takai et al., 2012; Jelassi et al., 2013; Barden et al., 2014; Takai et al., 2014; Schmid et al., 2015; Gilbert et al., 2019)
Kidney	(Liu et al., 2015; Gilbert et al., 2019)
Colorectal	(Coutinho-Silva et al., 2005; Barden et al., 2009; Li et al., 2009; Kunzli et al., 2011; Bian et al., 2013; Barden et al., 2014; Barden et al., 2016; Qian et al., 2017; Gilbert et al., 2019; Zhang et al., 2019)
Gastric	(Barden et al., 2009; Barden et al., 2014; Calik et al., 2020)
Breast	(Slater et al., 2004b; Barden et al., 2009; Li et al., 2009; Tafani et al., 2010; Jelassi et al., 2011; Huang et al., 2013; Jelassi et al., 2013; Barden et al., 2014; Chadet et al., 2014; Zheng et al., 2014; Tan et al., 2015; Draganov et al., 2015; Xia et al., 2015; Barden et al., 2016; Park et al., 2016; Gilbert et al., 2019; Park et al., 2019)
Cutaneous squamous-cell and basal-cell carcinomas	(Greig et al., 2003; Slater and Barden, 2005; Barden et al., 2016; Yang et al., 2016; Gilbert et al., 2017)
Melanoma	(Slater et al., 2003; White et al., 2005; Shankavaram et al., 2009; Reinhold et al., 2012; Barden et al., 2014; Roger et al., 2015; Gilbert et al., 2019)
Leukemia and lymphoma	(Peng et al., 1999; Gu et al., 2000; Adinolfi et al., 2002; Wiley et al., 2002; Barden et al., 2003; Zhang et al., 2003; Zhang et al., 2004; Yoon et al., 2006; Barden et al., 2009; Constantinescu et al., 2010; Chong et al., 2010b; Chong et al., 2010b; Wang et al., 2011; Barden et al., 2014; Chen et al., 2014; Ledderose et al., 2016; Salaro et al., 2016; Salvestrini et al., 2017)
Neuroblastoma	(Kaiho et al., 1998; Schrier et al., 2002; Raffaghello et al., 2006; Sun, 2010; Gutierrez-Martin et al., 2011; Bianchi et al., 2014; Amoroso et al., 2015; Gomez-Villafuertes et al., 2015; Wilkaniec et al., 2017; Ulrich et al., 2018; Gilbert et al., 2019)
Glioma	(Wei et al., 2008; Tamajusuku et al., 2010; Ryu et al., 2011; Tafani et al., 2011; Gehring et al., 2012; Fang et al., 2013; Barden et al., 2014; Gehring et al., 2015; McLarnon, 2017; Ji et al., 2018; Bergamin et al., 2019; Kan et al., 2019)
Ovarian	(Barden et al., 2014; Vazquez-Cuevas et al., 2014; Gilbert et al., 2019)
Cervical and endometrial	(Feng et al., 2006; Feng et al., 2006; Li et al., 2006; Li et al., 2007; Barden et al., 2009; Barden et al., 2014)
Bladder	(Barden et al., 2009; Barden et al., 2014; Hu et al., 2016; Gilbert et al., 2019)
Papillary Thyroid	(Ruggeri et al., 2002; Solini et al., 2008; Barden et al., 2009; Li et al., 2009; Dardano et al., 2009; Gu et al., 2010; Barden et al., 2014; Kwon et al., 2014)
Pancreatic	(Kunzli et al., 2007; Mistafa and Stenius, 2009; Barden et al., 2014; Giannuzzo et al., 2015; Giannuzzo et al., 2016; Choi et al., 2018; Gilbert et al., 2019)
Bone (osteosarcoma, Ewing sarcoma, chondromyxoid fibroma)	(Gartland et al., 2001; Alqallaf et al., 2009; Barden et al., 2009; Liu and Chen, 2010; Adinolfi et al., 2012b; Barden et al., 2014; Giuliani et al., 2014; Zhang et al., 2019a)
Head and neck	(Barden et al., 2009; Barden et al., 2014; Bae et al., 2017)
Testicular	(Barden et al., 2009; Barden et al., 2014)
Esophageal	(Barden et al., 2009; Barden et al., 2014; Santos et al., 2017)
Trophoblastic	(Barden et al., 2009; Barden et al., 2014)
Mesothelioma	(Barden et al., 2009; Barden et al., 2014; Amoroso et al., 2016)

P2X7 ACTIVATION CAN OPEN A TRANSMEMBRANE MACROPORE

Evidence for ATP-induced dye uptake was first identified in the 1970s and 80s (Rozengurt and Heppel, 1975; Cockcroft and Gomperts, 1979; Steinberg et al., 1987) but it was only a decade later that P2X7, previously known as P2Z, was shown to play a key role in the formation of a transmembrane dye-permeable macropore (Surprenant et al., 1996; Virginio et al., 1999). The formation of a transmembrane pore is not believed to be limited exclusively to P2X7. Other ion channels including P2X2, P2X4, TRPV1 and ASICs have also been shown to increase conductance and allow molecules of <900 Da into the cell (Khakh and Lester, 1999; Li et al., 2015). However, while P2X2, P2X4, TRPV1, and ASICs undergo a "channel to pore dilatation" upon prolonged activation, P2X7 pore opening is almost instantaneous (within milliseconds) (Li et al., 2015; Pippel et al., 2017).

While demonstration of the P2X7 macropore has been described extensively *in vitro* (Di Virgilio et al., 2018b), opening of the macropore *in vivo* remains to be demonstrated. Opening of the macropore allows the flow of ions across the membrane leading to rapid membrane depolarization and ultimately to cell death. *In vitro* assessment of macropore opening is typically performed by measuring the increase in fluorescence associated with the influx of ethidium$^+$ (314 Da), YO-PRO-1^{2+} (375 Da), Lucifer yellow (444 Da), DAPI (277 Da) or the current associated with N-methyl-D-glucamine$^+$ (NMDG$^+$, 195 Da) influx (Di Virgilio et al., 2019). Given the significant risk that an open macropore may have on cell viability, the low sensitivity of P2X7 for ATP provides a first mechanism of protection for cells. Furthermore, P2X7 macropore opening has been shown to be reversible upon the removal of ATP within 10 to 15 min from initiation of stimulation in a mouse macrophage cell line, however reversibility may be cell type specific (Di Virgilio et al., 1988; Di Virgilio et al., 2018b). This reversible closure of the macropore can potentially provide a second mechanism to protect cells from P2X7-mediated cell death. Although the advantages of gated opening of the P2X7 pore are not well understood, such transient mechanisms could allow small molecules, such as peptides and micro-RNA, to enter or leave the cell (Di Virgilio et al., 1988) as observed for connexins and pannexins at cell to cell junctions (Esseltine and Laird, 2016). The demonstration that P2X7 is permeable to the large natural cation spermidine, a polyamine molecule involved in cell survival, supports this hypothesis (Harkat et al., 2017). Since opening of a high conductance transmembrane conduit has inevitable dire consequences on cell survival, it is likely that under more physiological conditions, localized opening of the P2X7 macropore occurs that will perturb cytoplasmic ion homeostasis at discrete sites only (Franceschini et al., 2015).

The mechanism(s) leading to macropore opening have been the subject of much controversy. Several hypotheses have been proposed to support P2X7 dilatation in response to sustained agonist stimulation and the involvement of associated proteins

such as pannexin-1 (Reviewed in (Di Virgilio et al., 2017; Di Virgilio et al., 2018b)). However, recent analysis of human and rat P2X7 electrophysiological activity in response to agonist shows that the macropore function is intrinsic to P2X7 and does not involve progressive pore dilatation (Harkat et al., 2017; Pippel et al., 2017). Extracellular ATP is present at concentrations in the low nanomolar range in healthy tissue but can increase dramatically upon tissue damage, inflammation or during tumor development to the tens to hundreds of micromolar range and reach the high activation threshold of 0.3 to 0.5mM for P2X7 (Di Virgilio, 2015; Morciano et al., 2017; Di Virgilio et al., 2018b). We demonstrated that antibodies raised against the E200 epitope of P2X7 bind specifically to cancer cell lines that are devoid of macropore function but that retain P2X7 ion channel function (Barden et al., 2003; Gilbert et al., 2019). In these cell lines, overexpressed wild type P2X7 rescues macropore function but is not detected by antibodies that target E200 supporting the specific exposure of E200 epitope by nfP2X7 (Gilbert et al., 2019). E200 specific antibodies were used to show that nfP2X7 is present intracellularly and can be released to the plasma membrane upon stimulation with KM11060, a small molecule corrector that increases the secretion rate of misfolded proteins to the membrane as shown previously for the Cystic Fibrosis Transmembrane Receptor (CFTR) mutants (Robert et al., 2008). nfP2X7 was also shown to be necessary for cell survival (Gilbert et al., 2019). Hence, these data suggest that to sustain growth without compromising survival, cancer cells expressing high levels of P2X7 have developed a mechanism to inhibit macropore function while retaining ion channel functionality (Gilbert et al., 2019).

Although mutations of *P2X7,* such as E496A, have been shown to abrogate the macropore (Gu et al., 2001; Ghiringhelli et al., 2009), we and others have demonstrated that P2X7 is expressed at the cell membrane in the absence of mutations such as E496A. This suggests that additional mechanisms are involved in protecting cancer cells (Gilbert et al., 2019). Indeed, several other mechanisms have been shown to modulate P2X7 macropore activity. These include splice variants (Benzaquen et al., 2019), N-glycosylation (Lenertz et al., 2010), ADP-ribosylation (Adriouch et al., 2008), proteolytic cleavage (Young et al., 2018), and interactions with binding partners or other P2X family members (Guo et al., 2007; Gu et al., 2009). They also include interactions with cholesterol in the membrane (Robinson et al., 2014; Karasawa et al., 2017), that may attenuate or abrogate P2X7 macropore formation (summarized in **Table 1**). One or more of these mechanisms has the potential to protect cells that possess P2X7 receptors in a high ATP microenvironment over a sustained time period.

P2X7 AS A SCAVENGER DRIVING PHAGOCYTOSIS

Further to its ability to open an ion channel and macropore, P2X7 has also been shown to act as a scavenger receptor promoting phagocytosis of nonopsonised targets such as apoptotic cells, latex beads and live or dead bacteria (Staphylococcus *aureus* and Escherichia *coli*) under serum free conditions (Gu B. et al., 2010; Gu et al., 2011; Gu et al., 2012). Binding of P2X7 to the nonmuscle myosin heavy chain (NMMHC-IIA) was shown to be required for the engulfment of particles (Gu B. et al., 2010; Gu et al., 2011). This function is inversely correlated with P2X7 macropore opening, which requires NMMHC-IIA dissociation from P2X7 upon ATP stimulation (Gu et al., 2009; Gu B. et al., 2010; Gu et al., 2011). P2X7 mediated phagocytosis was first demonstrated in monocytes and macrophages before being extended to microglia cells in the CNS where it plays a critical role in the clearance of nonopsonised particles and debris involved in neuro-inflammation (Wiley et al., 2011; Gu and Wiley, 2018; Janks et al., 2018). The ability of the P2X7-NMMHC-IIA complex to act as a scavenger suggests another P2X7-mediated role for macrophages infiltrated in the TME where P2X7 would recognize and promote the phagocytosis of apoptotic cells and potentially support the presentation of associated antigen to immune effector cells. Indeed, during inflammation and in the TME, the extracellular ATP concentration is significantly higher than in healthy tissues (Di Virgilio et al., 2018b). Under these conditions, P2X7 (as well as P2X4) stimulation potentiates phagocytosis by inflammatory macrophages (Zumerle et al., 2019).

DOWNSTREAM EFFECT OF P2X7 ACTIVATION

Several signaling pathways are activated downstream of P2X7 including hypoxia and proinflammatory pathways *via* hypoxia-inducible factor 1a (HIF-1α), nitric oxide synthase (NOS2), cyclooxygenase 2 (COX2) and acute phase protein pentraxin-3 (PTX3) (Tafani et al., 2010). Downstream effects of P2X7 activation also include survival and proliferation pathways such as the NF-κB (Tafani et al., 2010; Liu et al., 2011), NFATc1 (Adinolfi et al., 2009), Phosphoinositide 3-kinases (PI3Ks) (Bian et al., 2013; Amoroso et al., 2015), mitogen-activated kinases (MAPK) (Bradford and Soltoff, 2002; Amstrup and Novak, 2003), phospholipase A2, C and D (Humphreys and Dubyak, 1996; Coutinho-Silva et al., 2003; Andrei et al., 2004) acid sphingomyelinase (Bianco et al., 2009) and myc oncogene (Amoroso et al., 2015).

The regulation of downstream signaling pathways by P2X7 is defined at three levels. First, the concentration and duration of agonist exposure. While acute exposure to relatively high amounts of ATP has been shown to activate PI3K/AKT and AMPK-PRAS-40-mTOR signaling pathways to trigger tumor cell death (Bian et al., 2013), lower and chronic activation has been shown to activate the NOD-like receptor containing a pyrin (NLRP3) inflammasome pathway. Second, regulation may involve the interaction of P2X7 with other membrane proteins, such as other P2X family members or Pannexin 1. Third, interaction with scaffolding proteins associated with the cytoskeleton such as NMMHC-IIA (Gu et al., 2009). Indeed, the P2X7 receptor signaling complex has been shown to regulate the macropore and interact with the actin cytoskeleton

machinery (Kim et al., 2001). These three levels of regulation are involved in several mechanisms that are key to cancer progression.

Cell Death

The early discovery of the P2X7 macropore drove interest in P2X7 as a receptor capable of mediating cell death through disruption of intracellular homeostasis and cytolysis leading to necrosis (Zanovello et al., 1990; Surprenant et al., 1996). However, many reports have highlighted cell death mechanisms associated with P2X7 activation that are typical of an apoptotic pathway. These include membrane depolarization, redistribution of phosphatidylserine (PS) to the outer membrane, caspase 3, 8 and 9 activation and membrane blebbing (Zanovello et al., 1990; Ferrari et al., 1997a; Humphreys et al., 2000; Mackenzie et al., 2005). Interestingly, the recent resolution of the rat P2X7 structure has highlighted the presence of a putative phospholipid in a perpendicular position to the transmembrane helices in the middle of the plasma membrane that led McCarthy and colleagues to suggest that P2X7 might possess an intrinsic flippase-like function driving PS redistribution to the outer membrane (McCarthy et al., 2019).

Proliferation

The notion that the P2X7 receptor, together with mediating cytotoxic activity, also exerts a proliferative drive dates back to 1999, when in a pivotal paper from Baricordi and colleagues, it was demonstrated that transfection with the receptor conferred a proliferative advantage to leukaemic cell lines growing in the absence of serum (Baricordi et al., 1999). In the following years, the trophic activity of P2X7 was associated with an increase in mitochondrial function leading to an augmented production of intracellular ATP (Adinolfi et al., 2005) and, in general, to increased levels of calcium inside cellular stores (Adinolfi et al., 2009).

P2X7 calcium channel activity plays a central role in driving proliferation and is shared by the human splice variant P2X7B that retains ion channel activity while losing the ability to form a macropore (Adinolfi et al., 2010). When transfected in the osteosarcoma cell line Te85, P2X7B causes an increase in proliferation that is even greater than that attributable to transfection of P2X7A (Giuliani et al., 2014). Several calcium-related intracellular pathways involved in cell proliferation were shown to be activated by the P2X7 receptor. These include the JNK/MAPK, PI3K/AKT/myc and HIF1α-VEGF pathways (Adinolfi et al., 2012b; Adinolfi et al., 2015b; Di Virgilio et al., 2016; Di Virgilio and Adinolfi, 2017; Orioli et al., 2017). Among these, the nuclear factor of activated T cells (NFAT) pathway is one of the best characterized. NFAT is a calcium-calcineurin activated nuclear factor involved in the proliferation of several cell types including T lymphocytes where P2X7-dependent activation of NFAT was shown to facilitate clonal expansion and IL-2 secretion thus positively influencing lymphocytic growth (Yu et al., 2010). NFATc1 mediated pathway was also responsible for increased proliferation of HEK-293 (Adinolfi et al., 2009; Adinolfi et al., 2010) and Te85 cells transfected with both P2X7A and B isoforms (Giuliani et al., 2014) and was found to be upregulated in xenografts overexpressing the receptor (Adinolfi et al., 2012a).

Association between P2X7, proliferation and survival has been demonstrated in several primary cell types including osteoblasts (Grol et al., 2013; Agrawal et al., 2017; Yang et al., 2019), microglia (Monif et al., 2009), and $CD8^+$ memory T cells (Borges da Silva et al., 2018) where it supports physiological functions such as inflammatory responses and osteogenesis. However, increased P2X7 activity was also linked with increased proliferation and survival in many cancer cell types including pancreatic cancer (Giannuzzo et al., 2016), leukemia (Salaro et al., 2016) and glioma (Ji et al., 2018). Indeed, reports showing enhanced P2X7 expression in tumor tissues compared with normal tissue across many cancer types suggest a role for P2X7 during tumorigenesis (See section: The function and expression of P2X7 across cancer types below). Following the demonstration of the growth-promoting activity of P2X7 *in vitro*, it was also shown in several studies that P2X7 can influence *in vivo* tumor growth and that consequently, down-modulation of the receptor by silencing or pharmacological intervention can reduce tumor burden in preclinical models (Di Virgilio et al., 2018a). Adinolfi and colleagues first demonstrated the growth-promoting activity of P2X7 in four different tumor models in mice including HEK-293 cells and ACN neuroblastoma derived xenografts and syngeneic colon carcinoma and melanoma models (Adinolfi et al., 2012a). Several studies have confirmed these data and extended them, demonstrating that P2X7 blockade leads to a general reduction of tumor burden in neuroblastoma (Amoroso et al., 2015; Ulrich et al., 2018), mesothelioma (Amoroso et al., 2016), melanoma (Hattori et al., 2012; Adinolfi et al., 2015a), glioma (Ji et al., 2018), osteosarcoma (Zhang et al., 2019a) and myeloid leukemia (De Marchi et al., 2019a).

Autophagy

Autophagy is an important mechanism that maintains cell homeostasis by eliminating damaged cellular components and recycling them *via* metabolic pathways. By doing so, autophagy safeguards energy production under cellular stress such as starvation (White et al., 2015). Depending on the time of activation, autophagy can either help to prevent cancer or exert a tumor-promoting action (Mulcahy Levy and Thorburn, 2020). While data supporting P2X7 involvement in autophagy and cancer progression are limited, the receptor is known to act on several pathways related to autophagy including the pathway leading to autophagosome formation (Orioli et al., 2017). Indeed, P2X7 was shown to activate autophagosome-lysosome fusion by upregulating Beclin1 (Sun et al., 2015; Mawatwal et al., 2017) and LC3-II (Young et al., 2015; Fabbrizio et al., 2017; Mawatwal et al., 2017). In dystrophic myoblasts, P2X7-dependent activation of autophagy correlated with the ability to form the macropore but not calcium influx-induced signaling (Young et al., 2015). The first demonstrations of P2X7-mediated trophic activity were obtained by growing cells under serum starvation where autophagy is typically induced (Baricordi et al., 1999; Adinolfi et al., 2005). Cancer cells cultured under these conditions were found to upregulate P2X7 expression suggesting that autophagy

can drive P2X7 overexpression and consequent trophic activity (Gomez-Villafuertes et al., 2015).

Chemotherapy-induced autophagy can be central in the modulation of the anticancer immune response (Kang et al., 2020). In particular, autophagy is a key driver in immunogenic cell death (ICD), a form of cancer cell death, which can elicit a potent immune response mediated by dendritic cells (DCs) and $CD8^+$ T lymphocytes that can eradicate residual tumor cells and prevents metastasis formation following a first therapeutic intervention (Galluzzi et al., 2017). Several classes of chemotherapeutics such as anthracyclines and oxaliplatin, are known to cause the induction of ICD (Galluzzi et al., 2017). Before ICD takes place, premortem autophagy is required for the release of ATP in the TME where ATP attracts antigen-presenting cells *via* P2Y2 receptors (Michaud et al., 2011; Ma et al., 2013) and down-regulates Treg infiltration (Pietrocola et al., 2016), to favor an antitumor immune response. Although a clear correlation between P2X7 activation and ICD in the TME is not available, it was recently demonstrated that P2X7 expression modulates ATP levels in the TME affecting CD73 and CD39 ectonucleotidase and reducing Treg levels and fitness (De Marchi et al., 2019). P2X7 systemic blockade activated an ICD–like mechanism in cancer cells leading to tumor growth reduction and activation of antitumor responses while reducing the tumor-promoting inflammatory cytokine IL-1β (De Marchi et al., 2019).

Role of P2X7 in the Immune Response

Analysis of data from P2X7 small molecule inhibitors and genetic knock out of *P2RX7* has demonstrated the key role played by P2X7 in inflammation and immunity (Di Virgilio et al., 2017; Adinolfi et al., 2018; Adinolfi et al., 2019) and, as a consequence, in chronic inflammatory diseases such as Duchenne muscular dystrophy (Ferrari et al., 1994; Sinadinos et al., 2015), rheumatoid arthritis (Stock et al., 2012) and inflammatory bowel disease (Arulkumaran et al., 2011; Eser et al., 2015). In the TME, the permanent release of DAMPs including ATP, high mobility group box 1 (HMGB1) and calreticulin (CRT) drives chronic inflammatory settings that are supplemented by immunosuppressive signals leading to a dampening of immune effector cell responses (Galluzzi et al., 2017). By responding to ATP proinflammatory signals, P2X7 counterbalances the potent immunosuppressive signaling driven by the ATP degradation product, adenosine (Li et al., 2019). In the following section, we describe the role of P2X7 in inflammation and immunity and use this as a foundation to define the role of P2X7 in the TME.

ATP, released through pannexin-1 hemichannels leads to activation of P2X7 as well as other P2 receptors including P2X1, P2X4, P2X5 and P2Y6 and is associated with the activation of T-cells (Wang et al., 2004; Overes et al., 2008; Schenk et al., 2008; Tsukimoto et al., 2009; Woehrle et al., 2010). However, the role of P2X7 in response to extracellular ATP appears to be central to the proinflammatory response including the stimulation of $CD4^+$ and $CD8^+$ effector T cells (Aswad et al., 2005; Schenk et al., 2008), stimulation of natural killer T cells (Beldi et al., 2008), inhibition of differentiation in type 1 regulatory cells, promotion of Treg cell death (Aswad et al., 2005; Schenk et al., 2011; Figliuolo et al., 2017) and the differentiation of inflammatory Th17 lymphocytes (Atarashi et al., 2008; Pandolfi et al., 2016). P2X7 was also found to collaborate with P2Y2 to promote the chemotaxis of myeloid cells including macrophages, neutrophils and the recruitment and activation of DCs (Idzko et al., 2002; Pelegrin et al., 2008; Savio et al., 2017). P2X7 activation also promotes the immunosuppressive role of myeloid-derived suppressor cells (MDSCs) infiltrated in the TME by stimulating the release of reactive oxygen species (ROS), arginase 1 (ARG1) and transforming growth factor-β1 (TGF-β1) (Bianchi et al., 2014).

At the molecular level, P2X7 plays a central role in the assembly and maturation of the NLRP3 inflammasome leading to the activation of caspase 1 followed by the cleavage of pro-interleukin 1 beta (pro-IL-1β) into mature IL-1β before IL-1β is released by macrophages and other immune cells (Ferrari et al., 1997b). Direct interaction of P2X7 with the components of the inflammasome including the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and the NOD-like receptor family (NLR) has been shown in neurons, astrocytes and microglial cells (Silverman et al., 2009; Minkiewicz et al., 2013; Franceschini et al., 2015). However, following opening of the P2X7 ion channel in response to the binding of ATP, it is the depletion of cytoplasmic K^+ that is the main driver leading to inflammasome activation (Sanz and Di Virgilio, 2000; Munoz-Planillo et al., 2013). While other channels also mediate K^+ efflux in response to ATP (Di et al., 2018), P2X7 activated K^+ efflux together with Ca^{2+} influx remain central to inflammasome activation and the secretion of cytokines and chemokines. Among these, IL-1β is a key proinflammatory cytokine secreted in response to P2X7 activation of the inflammasome (Perregaux and Gabel, 1998; Perregaux et al., 2000; Solle et al., 2001). Other cytokines and chemokines are also secreted by a variety of cell types. These include IL-6 (Solini et al., 1999; Kurashima et al., 2012; Shieh et al., 2014), IL-8 (Wei et al., 2008), IL-18 (Perregaux et al., 2000), tumor necrosis factor alpha (TNFα) (Ferrari et al., 2000; Shieh et al., 2014), CC-chemokine ligand 2 (CCL2 also named MCP-1) (Panenka et al., 2001; Kurashima et al., 2012; Shieh et al., 2014), CCL3 (Kataoka et al., 2009), CCL7 (Kurashima et al., 2012), CXCL2 (Shiratori et al., 2010; Kurashima et al., 2012), and prostaglandin E2 (PGE2) (Panupinthu et al., 2008; Barbera-Cremades et al., 2012). P2X7 also triggers activation and shedding of matrix metalloproteases (MMPs) from peripheral-blood mononuclear cells (PBMCs) (Gu et al., 1998; Gu and Wiley, 2006; Young et al., 2018). It also has been suggested that P2X7 plays a role in the resolution of inflammation through secretion of the anti-inflammatory cytokines IL-10 and TGFβ and proteins such as annexin A1 (Chessell et al., 2005; Bianchi et al., 2014; Moncao-Ribeiro et al., 2014; de Torre-Minguela et al., 2016). However, other reports have demonstrated P2X7 mediated inhibition of soluble HLA-G and IL-10 secretion by monocytes (Rizzo et al., 2009) and the negative modulation of HLA-G in women affected with herpes virus 6 A (Pegoraro et al., 2020).

P2X7 may cooperate in mediating the response to pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) through a potential LPS binding motif in the P2X7 C-terminal tail (Denlinger et al., 2001). LPS activation of TLR receptor also stimulates the production of pro-IL-1β, which is then processed by caspase-1 following P2X7 and NLRP3 inflammasome activation (Walev et al., 1995). Similarly, TLR2 and 4 were found to cooperate with P2X4 and P2X7 when activated by biglycan, a ubiquitous leucine-rich repeat proteoglycan found in the extracellular matrix (Babelova et al., 2009). P2X7 was shown to interact with the myeloid differentiation primary response 88 (MyD88) to activate the NF-kB signaling pathway (Liu et al., 2011). MyD88 mediates NF-kB signaling downstream of the TLR receptors and might mediate the cooperation between P2X7 and TLR signaling pathways. P2X7 together with CD14 as a coreceptor was found to support LPS binding and the internalization of P2X7 (Dagvadorj et al., 2015). Overall, these studies support a model whereby activation of TLR2 and 4 cooperate with P2X7 to drive chronic inflammatory settings. While ATP, PAMPs and biglycan were found to be key ligands of P2X7 and TLR receptors, in the TME, DAMPs such as ATP and HMGB1, a ligand for TLR4, are also present in significant concentrations and capable of driving the chronic inflammatory settings described above (Galluzzi et al., 2017).

Role of the P2X7 in Immunometabolism

Metabolic reprogramming and the maintenance of mitochondrial fitness are increasingly recognized as key factors in T lymphocyte differentiation and effector functions (Borges da Silva et al., 2018; Bailis et al., 2019). Generation of long-lived T memory cells depends on efficient oxidative phosphorylation and fatty acid oxidation, while effector T cells are mainly dependent on glycolysis (Patel and Powell, 2017). Therefore, mitochondria have a central role in directing T cell functions. The receptors and pathways mediating the regulation of mitochondrial metabolism modulation in T cells are still poorly characterized, however it is increasingly clear that the P2X7 receptor plays an important role. P2X7 has long been known as a prototypic cytotoxic receptor (Di Virgilio et al., 1998), but its tonic activity also supports healthy mitochondrial metabolism, enhances ATP production *via* the respiratory chain and promotes cell growth (Adinolfi et al., 2005; Adinolfi et al., 2009). The specific role of P2X7 in supporting metabolic fitness of long-lived T memory cells has now been demonstrated (Borges da Silva et al., 2018). Stimulation of the P2X7 receptor *via* extracellular ATP is crucial to promote memory $CD8^+$ T cell generation and long-term survival that is mediated mainly by a trophic effect on mitochondrial metabolism and the related increase in ATP synthesis. These data support a previous report from Di Virgilio and coworkers showing that P2X7 receptor expression has a broad effect on immunometabolism through modulation of glycolysis (Amoroso et al., 2012). Indeed, aerobic glycolysis is also impaired in P2X7-deficient $CD8^+$ T lymphocytes (Borges da Silva et al., 2018). Thus, the P2X7 receptor may function as a "metabolic sensor" that links the DAMP extracellular ATP, to the intracellular energy-producing machinery (i.e., oxidative phosphorylation and glycolysis).

The "metabolic sensor" function of the P2X7 receptor is of particular relevance at inflammatory and tumor sites since the extracellular ATP concentration in the inflammatory or tumor microenvironments (IME and TME, respectively) is several-fold higher than in the healthy interstitium (Pellegatti et al., 2008; Di Virgilio et al., 2018a). The impact of such a high extracellular ATP concentration on the viability of tumor infiltrated lymphocytes due to opening of the macropore is not known. While it is possible that loss of viability may take place, the long-term presence of infiltrated lymphocytes suggests that modulation of abrogation of macropore function through one or more of the mechanisms described in **Table 1** is likely to take place in these cells. Although details of the mechanism whereby P2X7 'transduces' the information carried by high extracellular ATP is not fully known, key immune functions are significantly affected by P2X7 activation (Di Virgilio et al., 2017; Di Virgilio and Adinolfi, 2017). Most importantly, P2X7 is a major stimulus for Il-1β and IL-18 release, and therefore for the initiation of inflammation, potentiation of antigen presentation by DCs and an efficient immune response, including antitumor immune responses (Mutini et al., 1999; Di Virgilio et al., 2018a). Converging reports by multiple laboratories have confirmed the initial observation by Di Virgilio and coworkers of a potent P2X7-mediated growth-promoting and stimulatory effect on human T lymphocytes (Baricordi et al., 1996; Schenk et al., 2008; Yip et al., 2009; Yu et al., 2010; Danquah et al., 2016). P2X7 is upregulated in Treg and T follicular helper cells (Tfh) that are severely inhibited by its activation (Gavin et al., 2007; Taylor et al., 2008; Hale et al., 2013). This is hypothesized to have important implications for host-microbiota interaction in gut-associated secondary lymphoid organs where ATP released by bacteria down-modulates the activity of resident Tfh cells, thus reducing secretion of high affinity IgA and their binding to commensal bacteria (Di Virgilio et al., 2018c). Grassi and coworkers have shown that P2X7 deletion affects microbiota composition and, in consequence, host metabolic homeostasis (Perruzza et al., 2017; Proietti et al., 2019). This might be significant in view of the known role of microbiota in the pathogenesis of several types of cancer and the influence of microbiota on the response to immune checkpoint inhibitors (Gopalakrishnan et al., 2018; Zitvogel et al., 2018; Vivarelli et al., 2019).

P2X7 and Purinergic Signaling in Antitumor Immunity

The function of the P2X7 receptor in the TME is better understood in the context of the overall mechanisms that control the extracellular ATP concentration (Adinolfi et al., 2019). Of importance is the role of CD39 and CD73 ectonucleotidases, that hydrolyze ATP to adenosine (Vijayan et al., 2017). Adenosine has long been considered one of the main drivers of immunosuppression (Antonioli et al., 2013). Yet, recent data demonstrates that the P2X7, CD39, and CD73 axis also plays a significant role in the modulation of the TME

immune component. P2X7 activation can be promoted by targeting CD39, with beneficial effects on antitumor responses that cannot be explained merely by the inhibition of adenosine generation (Li et al., 2019). The use of P2X7 targeted small molecule inhibitors and *P2RX7* genetic knock out in a murine melanoma model leads to modulation of CD39 and CD73 expression levels on several immune cell populations including Tregs, $CD4^+$ effector lymphocytes, macrophage and DCs (De Marchi et al., 2019). P2X7 null mice show a decrease in tumor-infiltrated $CD8^+$ T cells and an increase in Tregs overexpressing the fitness markers PD-1, OX40, and CD73. This outcome correlated with a decrease of extracellular ATP levels. In contrast, systemic inhibition of P2X7 with the antagonist A740003 did not affect the number of tumor-infiltrated $CD8^+$ and Treg lymphocytes but increased the number of $CD4^+$ effector T cells. A reduced expression of CD39 and CD73 was also observed on $CD4^+$ T cells and in DCs highlighting the importance of the crosstalk between P2X7, CD39, and CD73 in these cell types (De Marchi et al., 2019). The collaboration between P2X7 and CD39 was further demonstrated in two recent studies investigating the antitumoral activity of a CD39 blocking antibody (Li et al., 2019; Yan et al., 2020). Blocking of CD39 led to reduced tumor burden and metastatic spread in several murine models. Anti-CD39 antitumoral activity required the activation of P2X7 on immune cells as P2X7-mediated activation of the NLRP3 inflammasome led to IL-18 release by myeloid cells and induction of pyroptosis (Li et al., 2019; Yan et al., 2020). These data demonstrate that P2X7 collaboration with CD39 plays a central role in the regulation of anti-tumor immunity.

Membrane Blebbing

Membrane blebbing consists of plasma membrane sections protruding and retracting to create protrusions at the cell surface. Although the mechanism leading to membrane blebbing is not fully understood, P2X7 is one of the proteins that have been shown to initiate this mechanism upon sustained ATP stimulation for several minutes (MacKenzie et al., 2001). ATP mediated opening of the P2X7 macropore leads to a large Ca^{2+} influx that can induce membrane blebbing. Multiple proteins are involved in this mechanism. These include the serine/threonine kinase ROCK I (Morelli et al., 2003), heat shock protein HSP90 that negatively regulates receptor-mediated blebbing (Adinolfi et al., 2003) and the epithelial membrane protein 2 (EMP-2) (Wilson et al., 2002). EMP-2 was shown to interact with the P2X7 C-terminal tail suggesting that P2X7 mediated blebbing requires opening of the macropore (Wilson et al., 2002). While membrane blebbing is commonly associated with cellular stress and the initiation of the apoptotic pathway, P2X7 may also be involved in triggering reversible membrane blebbing allowing leukocytes to migrate and invade through extracellular matrices (Qu and Dubyak, 2009). Finally, P2X7-mediated membrane blebbing has been proposed as an early step leading to shedding of microvesicles in the microenvironment (Qu and Dubyak, 2009).

Release of Microvesicles

Since the discovery that P2X7 is central to the release of the proinflammatory cytokine IL-1β by macrophages, there have been extensive investigations on how this mechanism occurs, since IL-1β belongs to the leaderless secretory protein group lacking the secretory signal necessary for conventional protein secretion through the endoplasmic reticulum route (Dinarello, 2002). These studies have shown that IL-1β secretion is instead mainly caused by P2X7 induced nonconventional pathways including microvesicle shedding, exosomes and modified lysosomes (MacKenzie et al., 2001; Ferrari et al., 2006; Pizzirani et al., 2007; Qu et al., 2007; Lopez-Castejon and Brough, 2011; Piccioli and Rubartelli, 2013). Other studies further showed involvement of P2X7 in microvesicles that release cytokines by fibroblasts and microglia (Solini et al., 1999; Bianco et al., 2005), HIV particles by macrophages (Graziano et al., 2015) and tissue factor by macrophages and DCs (Baroni et al., 2007; Moore and MacKenzie, 2007).

Release of ROS

During environmental stress such as ionizing radiation, UV, or heat exposure, increased ROS production mediates oxidative stress, damages cellular structures and initiates cell death. However, low levels of ROS can act as signaling molecules. In phagocytes, ROS are used as bactericides to complete the phagocytosis process (D'Autreaux and Toledano, 2007; Reczek and Chandel, 2015). Hence, cells need to control ROS levels tightly in order to meet their physiological needs, while preventing cell death (Dupre-Crochet et al., 2013). Production of ROS is mediated mainly by NADPH oxidases (NOXs), and the electron transport chain in mitochondria. During tumor development, oncogenic stimulation, increased metabolic activity and mitochondrial defects lead to increased ROS production forcing cancer cells to increase their antioxidant capacity to prevent cell death (Reczek and Chandel, 2015). Several reports have shown the induction of ROS production downstream of P2X7 activation [reviewed in (Guerra et al., 2007)]. They show that P2X7-mediated Ca^{2+} influx leads to activation of kinases that phosphorylate NADPH oxidases (NOXs) to activate the production of ROS (Moore and MacKenzie, 2009; Wang and Sluyter, 2013). However, there are contradictory reports on the nature of the pathway involved in the phosphorylation of NOXs. In microglial cells, p38 MAPK and PI3K but not ERK1/2 have been shown to mediate ROS production (Parvathenani et al., 2003). In macrophages, two studies have shown activation of ERK1/2 downstream of PKC, c-Src, Pyk2 but no involvement of PI3K or p38 MAPK (Lenertz et al., 2009; Martel-Gallegos et al., 2013) while Noguchi et al. reported activation of the ASK1, p38 MAPK signaling pathway is required for ROS production (Noguchi et al., 2008). Low levels of ATP stimulation increase mitochondrial Ca^{2+} content leading to hyperpolarization of mitochondrial potential and increased ATP production (Adinolfi et al., 2005). In line with these data, P2X7 was shown to drive the expression of glycolytic enzymes leading to increased glycolysis and oxidative phosphorylation that

sustains cancer cell growth in the absence of glucose and serum (Amoroso et al., 2012).

CONTROL OF P2X7 LOCALIZATION AS A MEAN TO MODULATE P2X7 FUNCTION

P2X7 has been shown to localize in several cellular compartments including the plasma membrane, endoplasmic reticulum (ER), lysosomes, and phagosomes (Kuehnel et al., 2009; Robinson and Murrell-Lagnado, 2013). In human monocytes and lymphocytes, P2X7 was found to be localized mainly intracellularly while differentiation of monocytes into macrophages led to P2X7 localizing to the plasma membrane (Hickman et al., 1994; Gu et al., 2000; Gudipaty et al., 2001). In contrast, in mouse microglia and macrophages, P2X7 localizes mainly at the plasma membrane (Boumechache et al., 2009). In cancer cells, Gilbert and colleagues showed that nfP2X7 colocalizes with the ER marker, calreticulin indicating that a misfolded form of the receptor may be retained intracellularly. This was further supported by the relocalization of this form to the plasma membrane upon treatment with small molecule corrector KM11060 that increases the rate of protein secretion (Gilbert et al., 2019). Interestingly, this increased membrane expression was also stimulated by high ATP concentrations (500 μM and above) that suggests that cells may be using ER to plasma membrane localization as a mean to regulate P2X7 functions (Gilbert et al., 2019).

Palmitoylation of cysteine residues at the hinge of the C-terminal tail was shown to be required for P2X7 to localize to the plasma membrane and associate with lipid rafts (Gonnord et al., 2009). While several reports have confirmed P2X7 interaction with lipid rafts in multiple cell types (Bannas et al., 2005; Garcia-Marcos et al., 2006; Barth et al., 2007), the localization of P2X7 in lipid rafts may not be exclusive. Garcia-Marcos et al. have identified two P2X7 pools located in both lipid raft and nonlipid raft membrane compartments (Garcia-Marcos et al., 2006). They further showed that the nonlipid raft P2X7 pool can open a nonselective cation channel while the lipid raft P2X7 pool only is able to activate phospholipase A2 (PLA_2) (Garcia-Marcos et al., 2006). Hence, the nature of the lipid content in the membrane surrounding P2X7 receptors can regulate receptor signaling and ultimately its function. Indeed, cholesterol was shown to inhibit P2X7 macropore function (Robinson et al., 2014; Karasawa et al., 2017) while sphingomyelin and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG) were shown to enhance macropore function (Karasawa et al., 2017). Similarly, phosphatidylinositol 4,5-bisphosphate (PIP2) was shown to modulate P2X7 ion channel function (Zhao et al., 2007). Although no direct interaction was found between P2X7 and PIP2, several residues in the C-terminal tail (R385, K387 and K395) were shown to be involved in PIP2 regulation. The I568N polymorphism also located in the P2X7 C-terminal tail was shown to impair P2X7 trafficking to the membrane (Wiley et al., 2003) while the H155Y polymorphism increased P2X7 localization to the plasma membrane (Bradley et al., 2011).

Evidence from several groups points to the existence of signaling cassettes located in the C-terminal tail of P2X7 which supports P2X7 trafficking to the plasma membrane (Denlinger et al., 2003; Wiley et al., 2003; Smart et al., 2003; Chaumont et al., 2004). Indeed, Lenertz have shown the presence of an ER retention/retrieval cassette in the P2X7 C-terminal tail near R576 (Lenertz et al., 2009). This cassette allows P2X7 monomers to be retained in the ER while being assembled into trimers. P2X7 channels are then addressed to the plasma membrane through the secretory pathway (Lenertz et al., 2009). A similar mechanism has already been reported for other multimeric membrane receptors such as the N-methyl-D-aspartate (NMDA), glutamate receptor and the γ-aminobutyric acid type B (GABAB) receptor (Michelsen et al., 2005). The section of P2X7 C-terminal tail overlapping with that cassette also was found to bind to phospholipids as well as LPS in a macrophage cell line thereby modulating receptor membrane localization and its capacity to signal through the MAP Kinase and NF-κB pathway (Denlinger et al., 2001; Liu et al., 2011).

The Function and Expression of P2X7 Across Cancer Types

The presence of P2X7 has been shown in a large number of studies using tumor-derived biopsies, cell lines, xenografts and syngenic murine models across multiple and diverse cancer types. The majority of these publications, which demonstrate the presence of P2X7, have not directly assessed functionality of the P2X7 macropore. Given the high concentration of ATP in solid tumors, present at levels capable of inducing P2X7 pore activation (Pellegatti et al., 2008) and thereby death, it is unlikely that cells expressing fully functional P2X7 could survive in the TME over extended time periods. Therefore, it is probable that cells expressing P2X7 within the TME need to express P2X7 predominantly in a form where pore activity is attenuated. Despite this attenuation of pore function, it is clear from a number of studies that the P2X7 expressed in these cancer cells retains significant signaling functionality and the ability to drive the formation, survival and metastatic potential of tumor cells as previously reported (Jelassi et al., 2011; Adinolfi et al., 2012a; Amoroso et al., 2015; De Marchi et al., 2019; Gilbert et al., 2019).

The P2X7 receptor is expressed on a number of cancer types including, but not limited to prostate, lung, kidney, colorectal, gastric, breast, cutaneous squamous-cell and basal-cell carcinomas, melanoma, leukemia, neuroblastoma, glioma, ovarian, cervical, bladder, papillary thyroid, pancreatic and bone cancer. This includes nfP2X7 (Barden et al., 2014; Gilbert et al., 2019). The key data demonstrating the expression and function of P2X7 on these cancer types is discussed below while **Table 2** provides a list of references per cancer type.

Prostate Cancer

In an immunohistochemistry (IHC) study looking at 116 prostate cancer biopsies using an affinity-purified polyclonal antibody to the E200 epitope of P2X7 (supplied by Biosceptre),

P2X7, in a nonpore functioning form, was identified in all malignant samples regardless of their stage or the age of the patient (Slater et al., 2004a). This was confirmed using a mouse monoclonal antibody (BPM09, supplied by Biosceptre) (Maianski et al., 2007). In another study, increased expression of nfP2X7 was observed as prostate disease progressed (Barden et al., 2016). P2X7 expression by IHC also was compared with the levels of prostate-specific antigen (PSA) in 174 prostate cancer biopsies (Slater et al., 2005). Increased nfP2X7 staining correlated with increased PSA levels indicating that P2X7 may provide a diagnostic biomarker candidate for early prostate cancer. These results were consistent with those of a separate study demonstrating increased P2X7 mRNA and protein expression in prostate tumor samples compared with normal tissue (Ravenna et al., 2009). Increased P2X7 expression correlates with expression of epidermal growth factor receptor (EGFR) and estrogen receptor (ER)α, which are well known drivers of cancer cell proliferation, suggesting that P2X7 might cooperate with these receptors to promote cell proliferation (Ravenna et al., 2009). These observations support the proposed role for P2X7 in cancer cell survival and proliferation (Adinolfi et al., 2009; Adinolfi et al., 2010). Indeed, functional P2X7 was shown to drive invasion and metastasis of prostate cancer cell lines stimulated by extracellular ATP (Ghalali et al., 2014; Qiu et al., 2014).

Lung Cancer

In a study analyzing P2X7 mRNA expression in 26 patients with nonsmall cell lung cancer (NSCLC), compared with 21 patients with chronic obstructive pulmonary disease (COPD) without signs of malignancy, higher P2X7 expression was observed in bronchoalveolar lavage derived cells of tumors with distant metastases (Schmid et al., 2015). P2X7 is also expressed in human NSCLC cell lines including A549, PC9 and H292 cells but not in the nonmalignant bronchial epithelial cells BEAS-2B (Barden et al., 2009; Takai et al., 2012; Jelassi et al., 2013; Takai et al., 2014). In H292 cells, inhibition or down-regulation of P2X7 abrogated TGF-β1 induced migration and actin remodeling. P2X7 is required for TGF-β1-induced exocytosis of ATP that then acts as a paracrine factor in lung cancer cell model. Overall, these data suggest a role for P2X7 in promoting invasion and the development of aggressive forms of lung cancer.

Kidney Cancer

Clear-cell renal cell carcinoma (ccRCC) is the most common form of renal cell carcinoma. In a study analyzing 273 ccRCC patients by IHC, P2X7 expression was correlated with the clinicopathologic features and cancer-specific survival (CSS) (Liu et al., 2015). Although intratumoral P2X7 expression was lower than peritumoral P2X7 expression, those patients with high intratumoral P2X7 expression had a worse prognosis. Overall, these data suggest that intratumoral P2X7 is involved in the progression of ccRCC. In contrast, the significant P2X7 expression found in peritumoral tissues may reflect P2X7 involvement in the TME. Further exploration of the cell type expressing P2X7 in ccRCC TME is now needed to better understand the nature of that involvement.

Colorectal and Gastric Cancer

P2X7 protein was identified by IHC with staining distributed throughout the cell in a small number of normal colorectal epithelia and colon adenocarcinomas (Li et al., 2009). Qian et al. demonstrated that high P2X7 expression correlated with tumor size, lymph nodes metastasis, TNM stage and was also associated with poor overall survival in a cohort of 116 colon carcinoma (Qian et al., 2017). These contradicting results suggest that there may be subtypes of colorectal cancer characterized by their level of P2X7 expression. Indeed, Zhang et al. analyzed normal tissue and colorectal cancer samples from 97 patients and found both P2X7 high and P2X7 low populations with P2X7 high population having increased metastasis and reduced survival (Zhang et al., 2019b). Both P2X7 and nfP2X7 were identified in human colorectal cancer epithelium although the nfP2X7 expression was much more significant (Barden et al., 2014; Gilbert et al., 2019). While nfP2X7 was identified in the human HT-29 and Colo-205 colon cancer cell lines (Barden et al., 2009), other reports have shown P2X7 expression in human HCT8, Caco-2, Colo-205, and murine MCA38 colon cancer cell lines and the latter cell line was found to possess functional P2X7 macropore (Coutinho-Silva et al., 2005; Kunzli et al., 2011; Bian et al., 2013). Analysis of P2X7 expression in 156 gastric cancers corelated P2X7 expression with tumor burden and poor survival suggesting that P2X7 may be involved in the progression of gastric cancer (Calik et al., 2020).

Breast Cancer

An IHC study analyzing nfP2X7 expression in 40 breast tumors of diverse histological subtypes demonstrated that nfP2X7 expression was absent in normal and hyperplastic breast epithelial samples while *in situ* or invasive lobular or ductal carcinoma expressed high levels of nfP2X7 (Slater et al., 2004b). Tumor cells from invasive carcinomas showed membrane staining as opposed to intracellular staining in *in situ* carcinomas. Therefore, nfP2X7 membrane staining may be stage-specific and nfP2X7 expression levels may help to distinguish between different stages of breast cancer. P2X7 mRNA and protein also were upregulated under hypoxic conditions in the noninvasive breast cancer cell line MCF-7 which has a nonfunctional P2X7 pore (Tafani et al., 2010; Chadet et al., 2014). In contrast, two independent studies looking at P2X7 expression showed a reduction of P2X7 staining in breast cancer versus normal tissue (Li et al., 2009; Huang et al., 2013). The antibodies used in these studies were raised against the C-terminal tail of the receptor and are therefore likely to mainly detect P2X7 variant A. These antibodies are unable to discriminate between functional P2X7 and nfP2X7. Hence, while discrepancies exist for the expression of P2X7 variant A in breast cancer, nfP2X7 appears to be specifically upregulated at the surface of breast cancer cells. Tan et al. reported higher P2X7 expression levels in the breast cancer tissues when compared with normal breast tissue (Tan et al., 2015). Furthermore, a positive correlation was observed between P2X7 expression and

the estrogen receptor (ER)+ by qRT-PCR, western blot and immunohistochemistry analysis.

Cutaneous Squamous-Cell and Basal-Cell Carcinomas

P2X7 is highly expressed both in nodular basal cell carcinomas (BCC) and in infiltrative BCC cells where it was shown to be present in some tumor cell nuclei (Greig et al., 2003). P2X7 expression was also found in the human squamous cell carcinoma (SCC) cell line A431, (Greig et al., 2003). nfP2X7 protein also was upregulated in 25 SCC compared to 20 keratoacanthomas (KA) (Slater and Barden, 2005). Using an affinity purified sheep polyclonal antibody to nfP2X7, systemic treatment of advanced cat SCC showed decreased lesion size (Barden et al., 2016). In a more recent study, the presence of nfP2X7 was shown in BCC and antibodies specific for nfP2X7 rather than fully functional P2X7 were shown to be a safe treatment for BCC with 65% of patients showing reduced lesion area, 20% showing stable tumor size and 15% with increased lesion area (Gilbert et al., 2017). Overall, these data support the targeting of nfP2X7 in BCC and SCC.

Melanoma

In an IHC study looking at 80 human melanoma biopsies, nfP2X7 protein was overexpressed in malignant tissues when compared with nonmalignant samples. P2X7 upregulation was also observed on keratinocytes of the epidermis surrounding the tumor (Slater et al., 2003). A study analyzing P2X7 in 14 human melanoma biopsies confirmed the upregulation of P2X7 in melanoma with over 75% of samples staining positively (White et al., 2005). Upregulation of P2X7 in melanoma samples was confirmed in cell lines at the mRNA level. Indeed, whole genome microarray screening of the NCI-60 cancer cell line panel has shown full length P2X7 upregulation as a hallmark of melanoma cell lines (Shankavaram et al., 2009; Reinhold et al., 2012; Roger et al., 2015). The human melanoma cell line A375 was found to express P2X7 variant A and to have a functional large pore (White et al., 2005).

Leukemia

P2X7 is upregulated in T-cell acute lymphoblastic leukemia and in murine erythroleukemia (MEL) cells (Constantinescu et al., 2010; Chen et al., 2014). In MEL cells, P2X7 was also shown to have a functional macropore (Constantinescu et al., 2010). P2X7 was also upregulated in pediatric leukemias (Chong et al., 2010a) as well as in human myeloid leukaemic cell lines F-36P and HL-60 (Yoon et al., 2006). In a separate study, P2X7 mRNA and protein were upregulated in 8 out of 11 cell lines, 69 out of 87 bone marrow mononuclear cell (BMMC) samples from leukemia patients and 9 out of 10 myelodysplastic syndrome (MDS) patients (Zhang et al., 2004). Furthermore, P2X7 also was significantly upregulated in acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL) (Zhang et al., 2004). P2X7 expression was found to be higher in AML subtypes having poor prognosis. Following standard induction, P2X7 was upregulated in a group with a low remission rate (Zhang et al., 2004). Increased P2X7 expression was found in lymphocytes from patients with the evolutive form of B-cell chronic lymphocytic leukemia (B-CLL) (Adinolfi et al., 2002). Furthermore, P2X7 expression correlated with the severity of B-CLL. However, the large molecular weight pore function was not assessed in this study (Adinolfi et al., 2002). In a separate study, antibodies targeting P2X7 were shown to bind to the surface of B-CLL cells. While the analysis of P2X7 membrane expression by flow cytometry suggests that the B-CLL populations tested are not fully uniform, data suggest that B-CLL populations with both functional and nonfunctional macropore can be identified (Gu et al., 2000). Overall, these data support a role for P2X7 in the progression of leukemia and the development of aggressive forms of the disease.

Neuroblastoma

In an IHC study, P2X7 was highly expressed in neuroblastoma irrespective of the tumor grade. P2X7 was also expressed in several neuroblastoma cell lines including ACN, GI-CA-N, HTLA-230, GI-ME-N, LAN-5, LAN-1, SK-N-BE-2, and SH-SY-5Y with cell surface staining characterized for at least ACN (Raffaghello et al., 2006). P2X7 stimulation *in vitro* with ATP did not induce apoptosis of neuroblastoma cells but instead stimulated their proliferation *via* the enhanced secretion of substance P, which suggests that these cells were nonfunctional for P2X7 macropore (Raffaghello et al., 2006). In an independent study, P2X7 mRNA expression was analyzed in 131 patients with neuroblastoma (Amoroso et al., 2015). High P2X7 expression correlated with poor prognosis while patients with low P2X7 expression levels had less aggressive tumors (Amoroso et al., 2015).

Glioma

Both mRNA and protein were detected in human glioma cell lines U-138MG, U-251MG, and M059J. P2X7 was shown to mediate cell death in response to ATP in glioma cells (Wei et al., 2008; Tamajusuku et al., 2010; Gehring et al., 2012; Fang et al., 2013). While U-138 MG and U-251 MG cells appeared to have a nonfunctional P2X7 pore, M059J glioma cells were reported as having functional receptor macropore (Gehring et al., 2012). P2X7 is also upregulated in mouse GL261 glioma cells (Bergamin et al., 2019). P2X7 was also upregulated in an *in vivo* model where rats received an intra-striatal injection of C6 glioma cells (Ryu et al., 2011). In these cells, P2X7 promoted chemotaxis *in vitro,* which suggests that P2X7 may play a role in the metastasis process of glioma (Ryu et al., 2011). In a separate study, Gehring et al. showed that high P2X7 expression was a good prognostic factor for glioma radiosensitivity and survival probability (Gehring et al., 2015). P2X7 was highly upregulated at the mRNA and protein level in tumor tissue as opposed to peritumoral and adjacent normal tissue (Tafani et al., 2011). P2X7 upregulation was also observed in cancer stem cells from GBM cultured under hypoxic conditions. This evidence supports the potential involvement of P2X7 in cancer stem cells (Tafani et al., 2011).

Ovarian Cancer

P2X7 expression was analyzed by IHC in nine human ovarian carcinoma biopsies and compared with ovarian surface

epithelium in healthy tissue (Vazquez-Cuevas et al., 2014). P2X7 was highly expressed in both healthy tissue and ovarian carcinoma (Vazquez-Cuevas et al., 2014). P2X7 also was found to be expressed in the human ovarian cancer cell lines SKOV-3 and CAOV-3 (Vazquez-Cuevas et al., 2014). Both P2X7 and nfP2X7 were expressed in ovarian cancer tissue (Barden et al., 2014; Gilbert et al., 2019).

Cervical Cancer

IHC analysis of squamous cell cancer of the cervix showed increased nfP2X7-specific membranous staining when compared to normal tissue (Barden et al., 2014). In contrast, Li et al. have reported a decreased expression of full length P2X7 in tissues of complex hyperplasia with atypia or endometrial adenocarcinoma compared to normal endometrium, simple hyperplasia or complex hyperplasia tissues (Li et al., 2007).

Bladder Cancer

Using Protein Pathway Array (PPA) to investigate the expression of 285 proteins and phosphoproteins in bladder urothelial cell carcinoma tissues and adjacent nontumor tissues, Hu et al. reported that expression of P2X7 was an independent factor, favorable for overall survival (Hu et al., 2016). Both P2X7 and nfP2X7 were confirmed to be expressed in bladder cancer tissue in separate IHC studies (Barden et al., 2014; Gilbert et al., 2019).

Papillary Thyroid Cancer

P2X7 mRNA was upregulated in 37 human papillary thyroid cancer (PTC) samples when compared with adjacent normal tissue (Solini et al., 2008). P2X7 protein upregulation was also confirmed by IHC in all malignant tissues tested including the classical and follicular forms of the disease (Solini et al., 2008). Adjacent normal tissue was devoid of P2X7 staining. P2X7 staining was found to be diffuse in the cytoplasm and intense at the cell surface of malignant thyrocytes (Solini et al., 2008). P2X7 mRNA and protein was also detected in FB1 and FB2 human thyroid cancer cell lines. In response to ATP, these cells showed a P2X7 dependent release of IL-6, a cytokine associated with aggressiveness of PTC (Ruggeri et al., 2002; Solini et al., 2008). P2X7 upregulation in PTC was confirmed by IHC in two independent studies (Li et al., 2009; Gu L. et al., 2010). P2X7 upregulation was shown to correlate with tumor growth, capsular infiltration and lymph node metastases (Gu L. et al., 2010; Kwon et al., 2014). Additional reports showed nfP2X7 expression in PTC tissue (Barden et al., 2009; Barden et al., 2014). Overall, these studies support a correlation between P2X7 and nfP2X7 expression and the development of aggressive PTC.

Pancreatic Cancer

P2X7 mRNA and protein was shown to be upregulated in chronic pancreatitis and pancreatic cancer tissue and cell lines *in vitro* and *in vivo* (Kunzli et al., 2007; Giannuzzo et al., 2015; Giannuzzo et al., 2016). P2X7 large pore functionality was not assessed in these studies. Other reports demonstrated both P2X7 and nfP2X7 expression in pancreatic cancer tissue (Barden et al., 2014; Gilbert et al., 2019).

Bone Cancer

P2X7 was upregulated in osteosarcoma, Ewing sarcoma, chondromyxoid fibroma as well as in bone cancer cell lines such as SaOs-2 and HOS (Gartland et al., 2001; Alqallaf et al., 2009; Liu and Chen, 2010). SaOs-2 cells have a functional P2X7 large pore (Alqallaf et al., 2009). Moreover, P2X7 upregulation plays a key role in several malignancies metastasising to the bone that supports an involvement of P2X7 in the host tissue during the metastatic process (reviewed in (Di Virgilio et al., 2009; Adinolfi et al., 2012b; Adinolfi et al., 2015a). P2X7B splice variant was upregulated in osteosarcoma and its expression was associated with cell proliferation (Giuliani et al., 2014).

THERAPEUTIC APPROACHES TAKEN TO TARGET P2X7

Several pharmaceutical organizations have engaged in the development of both small-molecules and biologics directed against P2X7 (Mehta et al., 2014; Baudelet et al., 2015; Jacobson and Muller, 2016) (**Table 3**). These developments can be seen in the increasing number of filed patents for P2X7 targeted treatment (Gunosewoyo and Kassiou, 2010; Sluyter and Stokes, 2011; Park and Kim, 2017; Pevarello et al., 2017). Early development of small molecule inhibitors targeted the P2X7 orthosteric site to compete ATP mediated P2X7 activation. However, the lack of specificity of these inhibitors led to the identification and development of several classes of inhibitors that bind to the inter-subunit allosteric pocket preventing ATP induced rotation of each subunit and closure of the turret (Karasawa and Kawate, 2016). Within this inter-subunit allosteric pocket, several point mutants including but not limited to F88A, D92A, F95A, and F103A were identified to play an important role in the mode of action of these inhibitors (Allsopp et al., 2017; Allsopp et al., 2018; Bin Dayel et al., 2019). Engagement of this allosteric pocket allowed progressive development of antagonists in the low nanomolar range (**Table 2**) while providing better selectivity for P2X7 against other P2X family members (Donnelly-Roberts et al., 2009a; Allsopp et al., 2017; Allsopp et al., 2018; Bin Dayel et al., 2019). Initial identification of lead candidates against the inter-subunit allosteric pocket revealed compounds with good activity against human P2X7 but inactive against the rat isoform making pharmacology studies difficult (Michel et al., 2008a; Michel et al., 2008b; Caseley et al., 2015). However, docking of these candidates in the inter-subunit allosteric pocket highlighted the importance of human F95 (L95 in rat P2X7) in developing pi-stacking interactions with inhibitors (Caseley et al., 2015; Allsopp et al., 2017; Allsopp et al., 2018).

Several of these small molecules including AZD9056 (AstraZeneca), CE-224,535 (Pfizer), EVT-401 (Evotec), and GSK1482160 (GlaxoSmithKline plc) have entered clinical studies to treat rheumatoid arthritis as well as other inflammatory conditions (**Table 2**). Although all clinical data

TABLE 3 | Small molecule inhibitors and biologics used against P2X7.

Molecule	Format	pEC50/pIC50 (method used)	Binding site/other targets	Development stage	Disease settings	References
Nonselective inhibitors						
Suramin	Small molecule	> 300 μM (change in current)	Orthosteric site; Binds to multiple other targets including several P2 receptor subtypes, several growth factors, human immunodeficiency virus (HIV) reverse transcriptase, vasoactive intestinal peptide receptors and G protein–coupled receptors.	In the clinic	Used as antiparasitic agent against trypanosomes infection. Tested in the clinic for the treatment of autism and hand foot and mouth disease. Tested as combination with chemotherapy in multiple solid and blood born tumors.	(Middaugh et al., 1992; Surprenant et al., 1996; Jacobson et al., 2002)
NF279 (Suramin analog)	Small molecule	20 μM (Ca^{2+} influx at 3min) 891 nM (Yo-Pro uptake)	Orthosteric site High affinity to $P2X_1$	Preclinical	N/A	(Donnelly-Roberts et al., 2009a)
MRS2159	Small molecule	1.7 μM (Ca^{2+} influx at 3min) 288 nM (Yo-Pro uptake)	High affinity to $P2X_1$	Preclinical	N/A	(Donnelly-Roberts et al., 2009a)
2_,3_-dialdehyde ATP (oxidized ATP)	Small molecule closely related to ATP	100–300 μM (change in current)	Orthosteric site	Preclinical	N/A	(Surprenant et al., 1996; Michel et al., 2000; Hibell et al., 2001)
pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS)	Small molecule	3.2 μM (Ca^{2+} influx at 3min) 1.2 μM (Yo-Pro uptake)	Orthosteric site	Preclinical	N/A	(Donnelly-Roberts et al., 2009a; Huo et al., 2018)
pyridoxal-5′-phosphate-6-(2′-naphthylazo-6′-nitro-4′,8′-disulphonate) (PPNDS)	Small molecule	407 nM (Ca^{2+} influx at 3min) 200 nM (Yo-Pro uptake)	Orthosteric site	Preclinical	N/A	(Donnelly-Roberts et al., 2009a)
KN-62	Small molecule	10 μM (Ca^{2+} influx at 3min) 214 nM (Yo-Pro uptake)	Inter-subunit allosteric pocket and central cavity involving D92, F103, T90, and V312 in human P2X7. High concentrations (≥1 μM) also block $Ca2^{+}$/calmodulin-dependent protein kinase II.	Preclinical	N/A	(Donnelly-Roberts et al., 2009a; Bin Dayel et al., 2019)
Brilliant Blue G (BBG)	Small molecule	< 100 μM (Ca^{2+} influx at 3min) 1.9 μM (Yo-Pro uptake)	Inter-subunit allosteric pocket and central cavity involving D92, F103, and V312 residues. Also blocks voltage-gated sodium channels.	Preclinical	N/A	(Donnelly-Roberts et al., 2009a; Bin Dayel et al., 2019)
Selective inhibitors						
A438079	Small molecule	123 nM (Ca^{2+} influx at 3min) 933 nM (Yo-Pro uptake)	Inter-subunit allosteric pocket involving F88, D92, T94, F95, and F103 residues.	Preclinical	N/A	(Nelson et al., 2006; Donnelly-Roberts et al., 2009a; Allsopp et al., 2018)
A740003	Small molecule	44 nM (Ca^{2+} influx at 3min) 93 nM (Yo-Pro uptake)	Inter-subunit allosteric pocket involving F88, D92, T94, F95 and F103, M105 and V312 residues.	Preclinical	N/A	(Honore et al., 2006; Donnelly-Roberts et al., 2009b; Karasawa and Kawate, 2016; Allsopp et al., 2018)
A804598	Small molecule	11 nM (Ca^{2+} influx)	Inter-subunit allosteric pocket involving F88, F95, F103, M105, and F108 residues.	Preclinical	N/A	(Donnelly-Roberts et al., 2009b; Karasawa and Kawate, 2016)

(Continued)

TABLE 3 | Continued

Molecule	Format	pEC50/pIC50 (method used)	Binding site/other targets	Development stage	Disease settings	References
A839977	Small molecule	20–150 nM (Ca^{2+} influx and Yo-Pro uptake)	Not known	Preclinical	N/A	(Honore et al., 2009)
AZ10606120	Small molecule	10 - 200 nM (Ethudium uptake and Ca^{2+} influx)	Inter-subunit allosteric pocket involving residues 73 to 79, T90, T94, F103, and V312 residues.	Preclinical	N/A	(Michel and Fonfria, 2007; Karasawa and Kawate, 2016; Allsopp et al., 2017)
AZ11645373	Small molecule	10–20 nM (change in current and Yo-Pro uptake)	Inter-subunit allosteric pocket involving L83, S86, F88, D92, T94, F95, P96, F108, I310, and V312 residues.	Preclinical	N/A	(Stokes et al., 2006; Caseley et al., 2015; Bin Dayel et al., 2019)
AZD9056	Small molecule	12 nM (IL-1β secretion)	Not known	Evaluated in a Phase 2b against rheumatoid arthritis. Clinical trial number: NCT00520572	Rheumatoid arthritis	(Keystone et al., 2012; McInnes et al., 2014)
GSK1482160	Small molecule	Kd=5.09 ± 0.98 nmol/l, Ki=2.63 ± 0.6 nmol/l ([11C] GSK1482160 binding to HEK293-hP2X7 live cells)	noncompetitive, negative allosteric modulators	Phase 1: First Time in Human Study Evaluating the Safety, Tolerability, Pharmacokinetics, Pharmacodynamics and the Effect of Food of Single Assending Doses of GSK1482160. Clinical trial number: NCT00849134	Pain from inflammatory disease such as arthritis	(Di Virgilio et al., 2017; Han et al., 2017)
GW791343	Small molecule (positive modulator)	Negative allosteric modulator of human P2X7 but positive allosteric modulator of rat P2X7 at high concentrations.	Inter-subunit allosteric pocket involving F103 and V312 residues.	Preclinical	N/A	(Michel et al., 2008a; Karasawa and Kawate, 2016)
CE-224535 (Pfizer)	Small molecule	4 nM (Yo-Pro uptake) 1 nM (IL-1β secretion)	noncompetitive, negative allosteric modulators	Phase 2a: Efficacy and safety of CE-224,535, an antagonist of P2X7 receptor, in treatment of patients with rheumatoid arthritis inadequately controlled by methotrexate. Clinical trial number: NCT00628095;	Rheumatoid arthritis	(Duplantier et al., 2011; Di Virgilio et al., 2017)
EVT-401 (Evotec)	Small molecule	Not known	Not known	Phase 1 in healthy volunteers to investigate the safety, tolerability, pharmacokinetics and pharmacodynamics of EVT-401.	Inflammatory conditions such as Rheumatoid Arthritis	M.G. Kelly, J. Kincaid, Bicycloheteroaryl compounds as P2X7 modulators and uses thereof, US 7297700, 2007. Evotec Announces the Successful Completion of the First Phase 1 Study with EVT 401: https://www.evotec.com/en/invest/news–announcements/press-releases/p/evotec-announces-the-successful-completion-of-the-first-phase-i-study-with-evt-401-an-oral-p2x7-receptor-antagonist—very-

(Continued)

TABLE 3 | Continued

Molecule	Format	pEC50/pIC50 (method used)	Binding site/other targets	Development stage	Disease settings	References
						good-safety-profile-and-confirmed-on-target-activity-4447
JNJ47965567	Small molecule	5 nM (Ca^{2+} influx)	Inter-subunit allosteric pocket involving F88, F103, M105, F108 and V312 residues.	Preclinical	N/A	(Bhattacharya et al., 2013; Karasawa and Kawate, 2016)
JNJ42253432	Small molecule	20nM (Ca^{2+} influx)	Not known	Preclinical	N/A	(Lord et al., 2014)
JNJ54232334	Small molecule	0.3 nM against human P2X7, 32 nM against rat P2X7 (Ca^{2+} influx)	Not known	Preclinical	N/A	(Lord et al., 2015)
JNJ54140515	Small molecule	79 nM against rat P2X7 (Ca^{2+} influx)	Not known	Preclinical	N/A	(Lord et al., 2015)
JNJ54166060	Small molecule	4 nM (Ca^{2+} influx)	Not known Inhibits CYP3A (IC50 = 2 μM)	Preclinical	N/A	(Swanson et al., 2016)
Biologics						
L4	mAb	5nM (change in current)	Not known	Preclinical	N/A	(Buell et al., 1998b)
Hano43	Rat mAb	N/A	Not known	N/A	N/A	(Adriouch et al., 2008)
Hano44	Rat mAb	N/A	Paratope includes R151 in finger-like structure cysteine-rich region	N/A	N/A	(Adriouch et al., 2008)
K1G	Rabbit polyclonal Ab.	N/A	Paratope includes R151 in finger-like structure cysteine-rich region	N/A	N/A	(Adriouch et al., 2008)
1F11	Rat mAb	N/A	Not known	N/A	N/A	(Kurashima et al., 2012)
13A7 nanobody	Bivalent nanobody-Fc	0.5 nM (inhibit CD62L shedding in mouse cell line)	Bind finger-like structure cysteine-rich region (same region as Hano44) mouse P2X7. No binding to human P2X7	N/A	N/A	(Danquah et al., 2016)
14D5 nanobody (enhancer)	Bivalent nanobody-Fc	0.1 nM (enhanced CD62L shedding in mouse cell line)	Bind finger-like structure cysteine-rich region (same region as Hano44) mouse P2X7. No binding to human P2X7	N/A	N/A	(Danquah et al., 2016)
Dano1-Fc	Bivalent nanobody-Fc	0.2 nM (Ca^{2+} influx and DAPI uptake)	Not known	Preclinical	N/A	(Danquah et al., 2016)
BIL010t	Polyclonal Anti-nfP2X7 ointment	N/A	E200 peptide in P2X7 extracellular domain	Investigation of the Safety and Tolerability of BSCT (Anti-nf-P2X7) 10% Ointment (Completed, has results) Clinical trial number: NCT02587819	Basal Cell carcinoma (BCC)	(Gilbert et al., 2017)
BIL06v	Vaccine	N/A	E200 peptide in P2X7 extracellular domain	A Phase 1 Study to Evaluate the Safety, Tolerability, Immunogenicity and Antitumor Activity of BIL06v/Alhydrogel in Patients with Advanced Solid Tumors. (Active, not recruiting) Clinical trial number: ACTRN12618000838213	Solid Tumors	

are not yet available, no clear benefits to patients have been observed so far (Jacobson and Muller, 2016; Di Virgilio et al., 2017). A recent report of an anti-P2X7 bivalent nanobody-Fc with IC_{50} in the sub-nanomolar range developed by Ablynx (now Sanofi) appear to show promising preclinical efficacy in chronic inflammatory disease models (Danquah et al., 2016). As reports increasingly highlight the critical role of P2X7 and ATP in tumor biology and its microenvironment, the rationale for targeting P2X7 in cancer in single or combination therapies is becoming increasingly relevant (Di Virgilio et al., 2017; Li et al., 2019). Indeed, the recent report of the well-tolerated BIL010t, topical anti-P2X7 treatment of BCC (Biosceptre) revealed promising patient outcomes with 65% of patients treated showing reduced lesion area and 20% with stable disease (Gilbert et al., 2017). Assessment of safety and immunogenicity of an anti P2X7 vaccine (BIL06v – Biosceptre) used in patients with advanced solid tumors is ongoing in Australia.

CONCLUSION

Since the discovery of P2X7 as a cytotoxic receptor, studies have shown that the role of P2X7 in tumor biology and its microenvironment is much more complex than initially thought. Because of the significant involvement of P2X7 in the diverse mechanisms that drive tumor progression, this receptor is now believed to be of significant value as a target for the development of new cancer therapies. Preclinical and clinical evidence of P2X7-targeted therapeutics demonstrate the potential for these candidates as innovative cancer therapies. Furthermore, the interplay between the activities of P2X7 in tumor cell biology and antitumor immune responses indicates that P2X7 targeted therapies could also be of significant value when used in combination therapies. Therefore, additional studies, to better link the molecular mechanisms associated with P2X7 activation, signaling and cancer development, are now needed to drive highly innovative, safe and effective first in class therapeutics into the clinic.

AUTHOR CONTRIBUTIONS

Conception and design: RL, SM, EA, and FV. Writing, review, and/or revision of the manuscript: RL, EA, CH, MP, JB, FV, and SM.

FUNDING

CH and MP, together with Biopsceptre (UK) Limited, have received a CASE Award from the UK Research Council [Biotechnology and Biological Sciences Research Council (BBSRC) (Reference No. BB/M009513/1)] to support a Doctoral Training Partnership. Funder role: Function of P2X7 in healthy and diseased human skin.

EA is supported by the Italian Association for Cancer Research, IG grant IG22837. Funder role: support for the investigation of the purinergic-adenosinergic axis in inflammation leading to colon cancer development.

FV is supported by the following grants:

- Italian Association for Cancer Research, IG grant 22883. Funder role: support for the investigation of repurposing purinergic signalling for cancer therapy.

- Italian Association for Cancer Research IG grant n.18581. Funder role: support for the investigation of the role of the P2X7 receptor in reprogramming cancer cell metabolism.

- European Commission, Grant EU COST BM1406. Funder role: support for exchange of young investigators between laboratories involved in the investigation of P2X7 role in immune response.

- Royal Society Exchange Fellowship IES\R3\170196. Funder role: support for the exchange of investigators involved in the study of P2X7-dependent microvesicle shedding from mononuclear cells.

- Projects of National Interest (PRIN) Ministry of Education of Italy, grant n. 20178YTNWC. Funder role: support for investigation of the mechanism responsible for P2X7 receptor-mediated release of pro-inflammatory cytokines from mononuclear phagocytes.

REFERENCES

Abbracchio, M. P., and Burnstock, G. (1994). Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharmacol. Ther.* 64 (3), 445–475. doi: 10.1016/0163-7258(94)00048-4

Adinolfi, E., Melchiorri, L., Falzoni, S., Chiozzi, P., Morelli, A., and Tieghi, A. (2002). P2X7 receptor expression in evolutive and indolent forms of chronic B lymphocytic leukemia. *Blood* 99 (2), 706–708. doi: 10.1182/blood.V99.2.706

Adinolfi, E., Kim, M., Young, M. T., Di Virgilio, F., and Surprenant, A. (2003). Tyrosine phosphorylation of HSP90 within the P2X7 receptor complex negatively regulates P2X7 receptors. *J. Biol. Chem.* 278 (39), 37344–37351. doi: 10.1074/jbc.M301508200

Adinolfi, E., Callegari, M. G., Ferrari, D., Bolognesi, C., Minelli, M., and Wieckowski, M. R. (2005). Basal activation of the P2X7 ATP receptor elevates mitochondrial calcium and potential, increases cellular ATP levels, and promotes serum-independent growth. *Mol. Biol. Cell* 16 (7), 3260–3272. doi: 10.1091/mbc.E04-11-1025

Adinolfi, E., Callegari, M. G., Cirillo, M., Pinton, P., Giorgi, C., and Cavagna, D. (2009). Expression of the P2X7 receptor increases the Ca2+ content of the endoplasmic reticulum, activates NFATc1, and protects from apoptosis. *J. Biol. Chem.* 284 (15), 10120–10128. doi: 10.1074/jbc.M805805200

Adinolfi, E., Cirillo, M., Woltersdorf, R., Falzoni, S., Chiozzi, P., and Pellegatti, P. (2010). Trophic activity of a naturally occurring truncated isoform of the P2X7 receptor. *FASEB J.* 24 (9), 3393–3404. doi: 10.1096/fj.09-153601

Adinolfi, E., Raffaghello, L., Giuliani, A. L., Cavazzini, L., Capece, M., and Chiozzi, P. (2012a). Expression of P2X7 receptor increases in vivo tumor growth. *Cancer Res.* 72 (12), 2957–2969. doi: 10.1158/0008-5472.CAN-11-1947

Adinolfi, E., Amoroso, F., and Giuliani, A. L. (2012b). P2X7 Receptor Function in Bone-Related Cancer. *J. Osteoporos* 2012, 637863. doi: 10.1155/2012/637863

Adinolfi, E., Capece, M., Franceschini, A., Falzoni, S., Giuliani, A. L., and Rotondo, A. (2015a). Accelerated tumor progression in mice lacking the ATP receptor P2X7. *Cancer Res.* 75 (4), 635–644. doi: 10.1158/0008-5472.CAN-14-1259

Adinolfi, E., Capece, M., Amoroso, F., De Marchi, E., and Franceschini, A. (2015b). Emerging roles of P2X receptors in cancer. *Curr. Med. Chem.* 22 (7), 878–890. doi: 10.2174/0929867321666141012172913

Adinolfi, E., Giuliani, A. L., De Marchi, E., Pegoraro, A., Orioli, E., and Di Virgilio, F. (2018). The P2X7 receptor: A main player in inflammation. *Biochem. Pharmacol.* 151, 234–244. doi: 10.1016/j.bcp.2017.12.021

Adinolfi, E., De Marchi, E., Orioli, E., Pegoraro, A., and Di Virgilio, F. (2019). Role of the P2X7 receptor in tumor-associated inflammation. *Curr. Opin. Pharmacol.* 47, 59–64. doi: 10.1016/j.coph.2019.02.012

Adriouch, S., Bannas, P., Schwarz, N., Fliegert, R., Guse, A. H., and Seman, M. (2008). ADP-ribosylation at R125 gates the P2X7 ion channel by presenting a covalent ligand to its nucleotide binding site. *FASEB J.* 22 (3), 861–869. doi: 10.1096/fj.07-9294com

Agrawal, A., Henriksen, Z., Syberg, S., Petersen, S., Aslan, D., and Solgaard, M. (2017). P2X7Rs are involved in cell death, growth and cellular signaling in primary human osteoblasts. *Bone* 95, 91–101. doi: 10.1016/j.bone.2016.11.011

Allard, B., Longhi, M. S., Robson, S. C., and Stagg, J. (2017). The ectonucleotidases CD39 and CD73: Novel checkpoint inhibitor targets. *Immunol. Rev.* 276 (1), 121–144. doi: 10.1111/imr.12528

Allsopp, R. C., Dayl, S., Schmid, R., and Evans, R. J. (2017). Unique residues in the ATP gated human P2X7 receptor define a novel allosteric binding pocket for the selective antagonist AZ10606120. *Sci. Rep.* 7 (1), 725. doi: 10.1038/s41598-017-00732-5

Allsopp, R. C., Dayl, S., Bin Dayel, A., Schmid, R., and Evans, R. J. (2018). Mapping the Allosteric Action of Antagonists A740003 and A438079 Reveals a Role for the Left Flipper in Ligand Sensitivity at P2X7 Receptors. *Mol. Pharmacol.* 93 (5), 553–562. doi: 10.1124/mol.117.111021

Alqallaf, S. M., Evans, B. A., and Kidd, E. J. (2009). Atypical P2X receptor pharmacology in two human osteoblast-like cell lines. *Br. J. Pharmacol.* 156 (7), 1124–1135. doi: 10.1111/j.1476-5381.2009.00119.x

Amoroso, F., Falzoni, S., Adinolfi, E., Ferrari, D., and Di Virgilio, F. (2012). The P2X7 receptor is a key modulator of aerobic glycolysis. *Cell Death Dis.* 3, e370. doi: 10.1038/cddis.2012.105

Amoroso, F., Capece, M., Rotondo, A., Cangelosi, D., Ferracin, M., and Franceschini, A. (2015). The P2X7 receptor is a key modulator of the PI3K/GSK3beta/VEGF signaling network: evidence in experimental neuroblastoma. *Oncogene* 34 (41), 5240–5251. doi: 10.1038/onc.2014.444

Amoroso, F., Salaro, E., Falzoni, S., Chiozzi, P., Giuliani, A. L., and Cavallesco, G. (2016). P2X7 targeting inhibits growth of human mesothelioma. *Oncotarget* 7 (31), 49664–49676. doi: 10.18632/oncotarget.10430

Amstrup, J., and Novak, I. (2003). P2X7 receptor activates extracellular signal-regulated kinases ERK1 and ERK2 independently of Ca2+ influx. *Biochem. J.* 374 (Pt 1), 51–61. doi: 10.1042/BJ20030585

Andrei, C., Margiocco, P., Poggi, A., Lotti, L. V., Torrisi, M. R., and Rubartelli, A. (2004). Phospholipases C and A2 control lysosome-mediated IL-1 beta secretion: Implications for inflammatory processes. *Proc. Natl. Acad. Sci. U. S. A.* 101 (26), 9745–9750. doi: 10.1073/pnas.0308558101

Antonioli, L., Blandizzi, C., Pacher, P., and Hasko, G. (2013). Immunity, inflammation and cancer: a leading role for adenosine. *Nat. Rev. Cancer* 13 (12), 842–857. doi: 10.1038/nrc3613

Arulkumaran, N., Unwin, R. J., and Tam, F. W. (2011). A potential therapeutic role for P2X7 receptor (P2X7R) antagonists in the treatment of inflammatory diseases. *Expert Opin. Invest. Drugs* 20 (7), 897–915. doi: 10.1517/13543784.2011.578068

Aswad, F., Kawamura, H., and Dennert, G. (2005). High sensitivity of CD4+CD25 + regulatory T cells to extracellular metabolites nicotinamide adenine dinucleotide and ATP: a role for P2X7 receptors. *J. Immunol.* 175 (5), 3075–383. doi: 10.4049/jimmunol.175.5.3075

Atarashi, K., Nishimura, J., Shima, T., Umesaki, Y., Yamamoto, M., and Onoue, M. (2008). ATP drives lamina propria T(H)17 cell differentiation. *Nature* 455 (7214), 808–812. doi: 10.1038/nature07240

Babelova, A., Moreth, K., Tsalastra-Greul, W., Zeng-Brouwers, J., Eickelberg, O., and Young, M. F. (2009). Biglycan, a danger signal that activates the NLRP3 inflammasome via toll-like and P2X receptors. *J. Biol. Chem.* 284 (36), 24035–24048. doi: 10.1074/jbc.M109.014266

Bae, J. Y., Lee, S. W., Shin, Y. H., Lee, J. H., Jahng, J. W., and Park, K. (2017). P2X7 receptor and NLRP3 inflammasome activation in head and neck cancer. *Oncotarget*. 8 (30), 48972–48982. doi: 10.18632/oncotarget.16903

Bailis, W., Shyer, J. A., Zhao, J., Canaveras, J. C. G., Al Khazal, F. J., and Qu, R. (2019). Distinct modes of mitochondrial metabolism uncouple T cell differentiation and function. *Nature* 571 (7765), 403–407. doi: 10.1038/s41586-019-1311-3

Bannas, P., Adriouch, S., Kahl, S., Braasch, F., Haag, F., and Koch-Nolte, F. (2005). Activity and specificity of toxin-related mouse T cell ecto-ADP-ribosyltransferase ART2.2 depends on its association with lipid rafts. *Blood* 105 (9), 3663–3670. doi: 10.1182/blood-2004-08-3325

Barbera-Cremades, M., Baroja-Mazo, A., Gomez, A. I., Machado, F., Di Virgilio, F., and Pelegrin, P. (2012). P2X7 receptor-stimulation causes fever via PGE2 and IL-1beta release. *FASEB J.* 26 (7), 2951–2962. doi: 10.1096/fj.12-205765

Barden, J. A., Sluyter, R., Gu, B. J., and Wiley, J. S. (2003). Specific detection of non-functional human P2X(7) receptors in HEK293 cells and B-lymphocytes. *FEBS Lett.* 538 (1-3), 159–162. doi: 10.1016/S0014-5793(03)00172-8

Barden, J., Gidley-Baird, A., Armstrong, A., Bean, P., Pilkington, G., and Sempf, F. (2009). Abstract #LB-153: Evaluation of non-functional P2X7 receptor as a potential pan cancer therapeutic and diagnostic target. *Cancer Res.* 69 (9).

Barden, J. A., Yuksel, A., Pedersten, J., Danieletto, S., and Delprado, W. (2014). Non-Functional P2X7: A Novel and Ubiquitous Target in Human Cancer. *J. Clin. Cell Immunol.* 5 (4), 237. doi: 10.4172/2155-9899.1000237

Barden, J. A., Gidley-Baird, A., Teh, L. C., Rajasekariah, G. H., Pedersen, J., and Christensen, N. I. (2016). Therapeutic Targeting of the Cancer-Specific Cell Surface Biomarker nfP2X7. *J. Clin. Cell. Immunol.* 7 (3), 432. doi: 10.4172/2155-9899.1000432

Baricordi, O. R., Ferrari, D., Melchiorri, L., Chiozzi, P., Hanau, S., and Chiari, E. (1996). An ATP-activated channel is involved in mitogenic stimulation of human T lymphocytes. *Blood* 87 (2), 682–690. doi: 10.1182/blood.V87.2.682.bloodjournal872682

Baricordi, O. R., Melchiorri, L., Adinolfi, E., Falzoni, S., Chiozzi, P., and Buell, G. (1999). Increased proliferation rate of lymphoid cells transfected with the P2X(7) ATP receptor. *J. Biol. Chem.* 274 (47), 33206–33208. doi: 10.1074/jbc.274.47.33206

Baroni, M., Pizzirani, C., Pinotti, M., Ferrari, D., Adinolfi, E., and Calzavarini, S. (2007). Stimulation of P2 (P2X7) receptors in human dendritic cells induces the release of tissue factor-bearing microparticles. *FASEB J.* 21 (8), 1926–1933. doi: 10.1096/fj.06-7238com

Barth, K., Weinhold, K., Guenther, A., Young, M. T., Schnittler, H., and Kasper, M. (2007). Caveolin-1 influences P2X7 receptor expression and localization in mouse lung alveolar epithelial cells. *FEBS J.* 274 (12), 3021–3033. doi: 10.1111/j.1742-4658.2007.05830.x

Baudelet, D., Lipka, E., Millet, R., and Ghinet, A. (2015). Involvement of the P2X7 purinergic receptor in inflammation: an update of antagonists series since 2009 and their promising therapeutic potential. *Curr. Med. Chem.* 22 (6), 713–729. doi: 10.2174/0929867322666141212120926

Beldi, G., Wu, Y., Banz, Y., Nowak, M., Miller, L., and Enjyoji, K. (2008). Natural killer T cell dysfunction in CD39-null mice protects against concanavalin A-induced hepatitis. *Hepatology* 48 (3), 841–852. doi: 10.1002/hep.22401

Benzaquen, J., Heeke, S., Janho Dit Hreich, S., Douguet, L., Marquette, C. H., and Hofman, P. (2019). Alternative splicing of P2RX7 pre-messenger RNA in health and diseases: Myth or reality? *BioMed. J.* 42 (3), 141–154. doi: 10.1016/j.bj.2019.05.007

Bergamin, L. S., Capece, M., Salaro, E., Sarti, A. C., Falzoni, S., and Pereira, M. S. L. (2019). Role of the P2X7 receptor in in vitro and in vivo glioma tumor growth. *Oncotarget* 10 (47), 4840–4856. doi: 10.18632/oncotarget.27106

Bhattacharya, A., Wang, Q., Ao, H., Shoblock, J. R., Lord, B., and Aluisio, L. (2013). Pharmacological characterization of a novel centrally permeable P2X7 receptor antagonist: JNJ-47965567. *Br. J. Pharmacol.* 170 (3), 624–640. doi: 10.1111/bph.12314

Bian, S., Sun, X., Bai, A., Zhang, C., Li, L., and Enjyoji, K. (2013). P2X7 integrates PI3K/AKT and AMPK-PRAS40-mTOR signaling pathways to mediate tumor cell death. *PloS One* 8 (4), e60184. doi: 10.1371/journal.pone.0060184

Bianchi, G., Vuerich, M., Pellegatti, P., Marimpietri, D., Emionite, L., and Marigo, I. (2014). ATP/P2X7 axis modulates myeloid-derived suppressor cell functions

in neuroblastoma microenvironment. *Cell Death Dis.* 5, e1135. doi: 10.1038/cddis.2014.109

Bianco, F., Pravettoni, E., Colombo, A., Schenk, U., Moller, T., and Matteoli, M. (2005). Astrocyte-derived ATP induces vesicle shedding and IL-1 beta release from microglia. *J. Immunol.* 174 (11), 7268–7277. doi: 10.4049/jimmunol.174.11.7268

Bianco, F., Ceruti, S., Colombo, A., Fumagalli, M., Ferrari, D., and Pizzirani, C. (2006). A role for P2X7 in microglial proliferation. *J. Neurochem.* 99 (3), 745–758. doi: 10.1111/j.1471-4159.2006.04101.x

Bianco, F., Perrotta, C., Novellino, L., Francolini, M., Riganti, L., and Menna, E. (2009). Acid sphingomyelinase activity triggers microparticle release from glial cells. *EMBO J.* 28 (8), 1043–1054. doi: 10.1038/emboj.2009.45

Bin Dayel, A., Evans, R. J., and Schmid, R. (2019). Mapping the Site of Action of Human P2X7 Receptor Antagonists AZ11645373, Brilliant Blue G, KN-62, Calmidazolium, and ZINC58368839 to the Intersubunit Allosteric Pocket. *Mol. Pharmacol.* 96 (3), 355–363. doi: 10.1124/mol.119.116715

Boldt, W., Klapperstuck, M., Buttner, C., Sadtler, S., Schmalzing, G., and Markwardt, F. (2003). Glu496Ala polymorphism of human P2X7 receptor does not affect its electrophysiological phenotype. *Am. J. Physiol. Cell Physiol.* 284 (3), C749–C756. doi: 10.1152/ajpcell.00042.2002

Borges da Silva, H., Beura, L. K., Wang, H., Hanse, E. A., Gore, R., and Scott, M. C. (2018). The purinergic receptor P2RX7 directs metabolic fitness of long-lived memory CD8(+) T cells. *Nature* 559 (7713), 264–268. doi: 10.1038/s41586-018-0282-0

Bos, J. L., Rehmann, H., and Wittinghofer, A. (2007). GEFs and GAPs: critical elements in the control of small G proteins. *Cell* 129 (5), 865–877. doi: 10.1016/j.cell.2007.05.018

Boumechache, M., Masin, M., Edwardson, J. M., Gorecki, D. C., and Murrell-Lagnado, R. (2009). Analysis of assembly and trafficking of native P2X4 and P2X7 receptor complexes in rodent immune cells. *J. Biol. Chem.* 284 (20), 13446–13454. doi: 10.1074/jbc.M901255200

Bradford, M. D., and Soltoff, S. P. (2002). P2X7 receptors activate protein kinase D and p42/p44 mitogen-activated protein kinase (MAPK) downstream of protein kinase C. *Biochem. J.* 366 (Pt 3), 745–755. doi: 10.1042/BJ20020358

Bradley, H. J., Baldwin, J. M., Goli, G. R., Johnson, B., Zou, J., and Sivaprasadarao, A. (2011). Residues 155 and 348 contribute to the determination of P2X7 receptor function via distinct mechanisms revealed by single-nucleotide polymorphisms. *J. Biol. Chem.* 286 (10), 8176–8187. doi: 10.1074/jbc.M110.211284

Brake, A. J., Wagenbach, M. J., and Julius, D. (1994). New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. *Nature* 371 (6497), 519–523. doi: 10.1038/371519a0

Buell, G. N., Talabot, F., Gos, A., Lorenz, J., Lai, E., and Morris, M. A. (1998a). Gene structure and chromosomal localization of the human P2X7 receptor. *Recept. Channels* 5 (6), 347–354.

Buell, G., Chessell, I. P., Michel, A. D., Collo, G., Salazzo, M., and Herren, S. (1998b). Blockade of human P2X7 receptor function with a monoclonal antibody. *Blood* 92 (10), 3521–3528. doi: 10.1182/blood.V92.10.3521

Burnstock, G. (2006). Purinergic signalling. *Br. J. Pharmacol.* 147 Suppl 1, S172–S181. doi: 10.1038/sj.bjp.0706429

Burnstock, G. (2014). Purinergic signalling: from discovery to current developments. *Exp. Physiol.* 99 (1), 16–34. doi: 10.1113/expphysiol.2013.071951

Cabrini, G., Falzoni, S., Forchap, S. L., Pellegatti, P., Balboni, A., and Agostini, P. (2005). A His-155 to Tyr polymorphism confers gain-of-function to the human P2X7 receptor of human leukemic lymphocytes. *J. Immunol.* 175 (1), 82–89. doi: 10.4049/jimmunol.175.1.82

Calik, I., Calik, M., Sarikaya, B., Ozercan, I. H., Arslan, R., and Artas, G. (2020). P2X7R as an independent prognostic indicator in gastric cancer. *Bosn. J. Basic Med. Sci.* 20 (2), 188–196. doi: 10.17305/bjbms.2020.4620

Caseley, E. A., Muench, S. P., Baldwin, S. A., Simmons, K., Fishwick, C. W., and Jiang, L. H. (2015). Docking of competitive inhibitors to the P2X7 receptor family reveals key differences responsible for changes in response between rat and human. *Bioorg. Med. Chem. Lett.* 25 (16), 3164–3167. doi: 10.1016/j.bmcl.2015.06.001

Chadet, S., Jelassi, B., Wannous, R., Angoulvant, D., Chevalier, S., and Besson, P. (2014). The activation of P2Y2 receptors increases MCF-7 breast cancer cells migration through the MEK-ERK1/2 signalling pathway. *Carcinogenesis* 35 (6), 1238–1247. doi: 10.1093/carcin/bgt493

Chaumont, S., Jiang, L. H., Penna, A., North, R. A., and Rassendren, F. (2004). Identification of a trafficking motif involved in the stabilization and polarization of P2X receptors. *J. Biol. Chem.* 279 (28), 29628–29638. doi: 10.1074/jbc.M403940200

Cheewatrakoolpong, B., Gilchrest, H., Anthes, J. C., and Greenfeder, S. (2005). Identification and characterization of splice variants of the human P2X7 ATP channel. *Biochem. Biophys. Res. Commun.* 332 (1), 17–27. doi: 10.1016/j.bbrc.2005.04.087

Chen, S., Feng, W., Yang, X., Yang, W., Ru, Y., and Liao, J. (2014). Functional expression of P2X family receptors in macrophages is affected by microenvironment in mouse T cell acute lymphoblastic leukemia. *Biochem. Biophys. Res. Commun.* 446 (4), 1002–1009. doi: 10.1016/j.bbrc.2014.03.048

Chessell, I. P., Hatcher, J. P., Bountra, C., Michel, A. D., Hughes, J. P., and Green, P. (2005). Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory and neuropathic pain. *Pain* 114 (3), 386–396. doi: 10.1016/j.pain.2005.01.002

Choi, J. H., Ji, Y. G., Ko, J. J., Cho, H. J., and Lee, D. H. (2018). Activating P2X7 Receptors Increases Proliferation of Human Pancreatic Cancer Cells via ERK1/2 and JNK. *Pancreas* 47 (5), 643–651. doi: 10.1097/MPA.0000000000001055

Chong, J. H., Zheng, G. G., Zhu, X. F., Guo, Y., Wang, L., and Ma, C. H. (2010a). Abnormal expression of P2X family receptors in Chinese pediatric acute leukemias. *Biochem. Biophys. Res. Commun.* 391 (1), 498–504. doi: 10.1016/j.bbrc.2009.11.087

Chong, J. H., Zheng, G. G., Ma, Y. Y., Zhang, H. Y., Nie, K., and Lin, Y. M. (2010b). The hyposensitive N187D P2X7 mutant promotes malignant progression in nude mice. *J. Biol. Chem.* 285 (46), 36179–36187. doi: 10.1074/jbc.M110.128488

Cockcroft, S., and Gomperts, B. D. (1979). ATP induces nucleotide permeability in rat mast cells. *Nature* 279 (5713), 541–542. doi: 10.1038/279541a0

Coddou, C., Yan, Z., Obsil, T., Huidobro-Toro, J. P., and Stojilkovic, S. S. (2011). Activation and regulation of purinergic P2X receptor channels. *Pharmacol. Rev.* 63 (3), 641–683. doi: 10.1124/pr.110.003129

Constantinescu, P., Wang, B., Kovacevic, K., Jalilian, I., Bosman, G. J., and Wiley, J. S. (2010). P2X7 receptor activation induces cell death and microparticle release in murine erythroleukemia cells. *Biochim. Biophys. Acta* 1798 (9), 1797–1804. doi: 10.1016/j.bbamem.2010.06.002

Costa-Junior, H. M., Sarmento Vieira, F., and Coutinho-Silva, R. (2011). C terminus of the P2X7 receptor: treasure hunting. *Purinerg. Signal* 7 (1), 7–19. doi: 10.1007/s11302-011-9215-1

Coutinho-Silva, R., Stahl, L., Raymond, M. N., Jungas, T., Verbeke, P., and Burnstock, G. (2003). Inhibition of chlamydial infectious activity due to P2X7R-dependent phospholipase D activation. *Immunity* 19 (3), 403–412. doi: 10.1016/s1074-7613(03)00235-8

Coutinho-Silva, R., Stahl, L., Cheung, K. K., de Campos, N. E., de Oliveira Souza, C., and Ojcius, D. M. (2005). P2X and P2Y purinergic receptors on human intestinal epithelial carcinoma cells: effects of extracellular nucleotides on apoptosis and cell proliferation. *Am. J. Physiol. Gastrointest. Liver Physiol.* 288 (5), G1024–G1035. doi: 10.1152/ajpgi.00211.2004

D'Autreaux, B., and Toledano, M. B. (2007). ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat. Rev. Mol. Cell Biol.* 8 (10), 813–824. doi: 10.1038/nrm2256

Dagvadorj, J., Shimada, K., Chen, S., Jones, H. D., Tumurkhuu, G., and Zhang, W. (2015). Lipopolysaccharide Induces Alveolar Macrophage Necrosis via CD14 and the P2X7 Receptor Leading to Interleukin-1alpha Release. *Immunity* 42 (4), 640–653. doi: 10.1016/j.immuni.2015.03.007

Danquah, W., Meyer-Schwesinger, C., Rissiek, B., Pinto, C., Serracant-Prat, A., and Amadi, M. (2016). Nanobodies that block gating of the P2X7 ion channel ameliorate inflammation. *Sci. Transl. Med.* 8 (366), 366ra162. doi: 10.1126/scitranslmed.aaf8463

Dardano, A., Falzoni, S., Caraccio, N., Polini, A., Tognini, S., and Solini, A. (2009). 1513A>C polymorphism in the P2X7 receptor gene in patients with papillary thyroid cancer: correlation with histological variants and clinical parameters. *J. Clin. Endocrinol. Metab.* 94 (2), 695–698. doi: 10.1210/jc.2008-1322

de Andrade Mello, P., Coutinho-Silva, R., and Savio, L. E. B. (2017). Multifaceted Effects of Extracellular Adenosine Triphosphate and Adenosine in the Tumor-Host Interaction and Therapeutic Perspectives. *Front. Immunol.* 8, 1526. doi: 10.3389/fimmu.2017.01526

De Marchi, E., Orioli, E., Dal Ben, D., and Adinolfi, E. (2016). P2X7 Receptor as a Therapeutic Target. *Adv. Protein Chem. Struct. Biol.* 104, 39–79. doi: 10.1016/bs.apcsb.2015.11.004

De Marchi, E., Orioli, E., Pegoraro, A., Sangaletti, S., Portararo, P., and Curti, A. (2019). The P2X7 receptor modulates immune cells infiltration, ectonucleotidases expression and extracellular ATP levels in the tumor microenvironment. *Oncogene* 38 (19), 3636–3650. doi: 10.1038/s41388-019-0684-y

de Torre-Minguela, C., Barbera-Cremades, M., Gomez, A. I., Martin-Sanchez, F., and Pelegrin, P. (2016). Macrophage activation and polarization modify P2X7 receptor secretome influencing the inflammatory process. *Sci. Rep.* 6, 22586. doi: 10.1038/srep22586

Denlinger, L. C., Fisette, P. L., Sommer, J. A., Watters, J. J., Prabhu, U., and Dubyak, G. R. (2001). Cutting edge: the nucleotide receptor P2X7 contains multiple protein- and lipid-interaction motifs including a potential binding site for bacterial lipopolysaccharide. *J. Immunol.* 167 (4), 1871–1876. doi: 10.4049/jimmunol.167.4.1871

Denlinger, L. C., Sommer, J. A., Parker, K., Gudipaty, L., Fisette, P. L., and Watters, J. W. (2003). Mutation of a dibasic amino acid motif within the C terminus of the P2X7 nucleotide receptor results in trafficking defects and impaired function. *J. Immunol.* 171 (3), 1304–1311. doi: 10.4049/jimmunol.171.3.1304

Di, A., Xiong, S., Ye, Z., Malireddi, R. K. S., Kometani, S., and Zhong, M. (2018). The TWIK2 Potassium Efflux Channel in Macrophages Mediates NLRP3 Inflammasome-Induced Inflammation. *Immunity* 49 (1), 56–65 e4. doi: 10.1016/j.immuni.2018.04.032

Di Virgilio, F., and Adinolfi, E. (2017). Extracellular purines, purinergic receptors and tumor growth. *Oncogene* 36 (3), 293–303. doi: 10.1038/onc.2016.206

Di Virgilio, F., Meyer, B. C., Greenberg, S., and Silverstein, S. C. (1988). Fc receptor-mediated phagocytosis occurs in macrophages at exceedingly low cytosolic Ca2+ levels. *J. Cell Biol.* 106 (3), 657–666. doi: 10.1083/jcb.106.3.657

Di Virgilio, F., Chiozzi, P., Falzoni, S., Ferrari, D., Sanz, J. M., and Venketaraman, V. (1998). Cytolytic P2X purinoceptors. *Cell Death Differ.* 5 (3), 191–199. doi: 10.1038/sj.cdd.4400341

Di Virgilio, F., Ferrari, D., and Adinolfi, E. (2009). P2X(7): a growth-promoting receptor-implications for cancer. *Purinerg. Signal* 5 (2), 251–256. doi: 10.1007/s11302-009-9145-3

Di Virgilio, F., Falzoni, S., Giuliani, A. L., and Adinolfi, E. (2016). P2 receptors in cancer progression and metastatic spreading. *Curr. Opin. Pharmacol.* 29, 17–25. doi: 10.1016/j.coph.2016.05.001

Di Virgilio, F., Dal Ben, D., Sarti, A. C., Giuliani, A. L., and Falzoni, S. (2017). The P2X7 Receptor in Infection and Inflammation. *Immunity* 47 (1), 15–31. doi: 10.1016/j.immuni.2017.06.020

Di Virgilio, F., Sarti, A. C., Falzoni, S., De Marchi, E., and Adinolfi, E. (2018a). Extracellular ATP and P2 purinergic signalling in the tumour microenvironment. *Nat. Rev. Cancer* 18 (10), 601–618. doi: 10.1038/s41568-018-0037-0

Di Virgilio, F., Schmalzing, G., and Markwardt, F. (2018b). The Elusive P2X7 Macropore. *Trends Cell Biol.* 28 (5), 392–404. doi: 10.1016/j.tcb.2018.01.005

Di Virgilio, F., Sarti, A. C., and Grassi, F. (2018c). Modulation of innate and adaptive immunity by P2X ion channels. *Curr. Opin. Immunol.* 52, 51–59. doi: 10.1016/j.coi.2018.03.026

Di Virgilio, F., Jiang, L. H., Roger, S., Falzoni, S., Sarti, A. C., and Vultaggio-Poma, V. (2019). Structure, function and techniques of investigation of the P2X7 receptor (P2X7R) in mammalian cells. *Methods Enzymol.* 629, 115–150. doi: 10.1016/bs.mie.2019.07.043

Di Virgilio, F. (2015). P2X receptors and inflammation. *Curr. Med. Chem.* 22 (7), 866–877. doi: 10.2174/0929867322666141210155311

Dinarello, C. A. (2002). The IL-1 family and inflammatory diseases. *Clin. Exp. Rheumatol.* 20 (5 Suppl 27), S1–13.

Donnelly-Roberts, D. L., Namovic, M. T., Han, P., and Jarvis, M. F. (2009a). Mammalian P2X7 receptor pharmacology: comparison of recombinant mouse, rat and human P2X7 receptors. *Br. J. Pharmacol.* 157 (7), 1203–1214. doi: 10.1111/j.1476-5381.2009.00233.x

Donnelly-Roberts, D. L., Namovic, M. T., Surber, B., Vaidyanathan, S. X., Perez-Medrano, A., and Wang, Y. (2009b). [3H]A-804598 ([3H]2-cyano-1-[(1S)-1-phenylethyl]-3-quinolin-5-ylguanidine) is a novel, potent, and selective antagonist radioligand for P2X7 receptors. *Neuropharmacology* 56 (1), 223–229. doi: 10.1016/j.neuropharm.2008.06.012

Draganov, D., Gopalakrishna-Pillai, S., Chen, Y. R., Zuckerman, N., Moeller, S., and Wang, C. (2015). Modulation of P2X4/P2X7/Pannexin-1 sensitivity to extracellular ATP via Ivermectin induces a non-apoptotic and inflammatory form of cancer cell death. *Sci. Rep.* 5, 16222. doi: 10.1038/srep16222

Dubyak, G. R. (2007). Go it alone no more–P2X7 joins the society of heteromeric ATP-gated receptor channels. *Mol. Pharmacol.* 72 (6), 1402–1405. doi: 10.1124/mol.107.042077

Duplantier, A. J., Dombroski, M. A., Subramanyam, C., Beaulieu, A. M., Chang, S. P., and Gabel, C. A. (2011). Optimization of the physicochemical and pharmacokinetic attributes in a 6-azauracil series of P2X7 receptor antagonists leading to the discovery of the clinical candidate CE-224,535. *Bioorg. Med. Chem. Lett.* 21 (12), 3708–3711. doi: 10.1016/j.bmcl.2011.04.077

Dupre-Crochet, S., Erard, M., and Nubetae, O. (2013). ROS production in phagocytes: why, when, and where? *J. Leukoc. Biol.* 94 (4), 657–670. doi: 10.1189/jlb.1012544

Eltzschig, H. K., Sitkovsky, M. V., and Robson, S. C. (2012). Purinergic signaling during inflammation. *N Engl. J. Med.* 367 (24), 2322–2333. doi: 10.1056/NEJMra1205750

Eser, A., Colombel, J. F., Rutgeerts, P., Vermeire, S., Vogelsang, H., and Braddock, M. (2015). Safety and Efficacy of an Oral Inhibitor of the Purinergic Receptor P2X7 in Adult Patients with Moderately to Severely Active Crohn's Disease: A Randomized Placebo-controlled, Double-blind, Phase IIa Study. *Inflammation Bowel Dis.* 21 (10), 2247–2253. doi: 10.1097/MIB.0000000000000514

Esseltine, J. L., and Laird, D. W. (2016). Next-Generation Connexin and Pannexin Cell Biology. *Trends Cell Biol.* 26 (12), 944–955. doi: 10.1016/j.tcb.2016.06.003

Fabbrizio, P., Amadio, S., Apolloni, S., and Volonte, C. (2017). P2X7 Receptor Activation Modulates Autophagy in SOD1-G93A Mouse Microglia. *Front. Cell Neurosci.* 11, 249. doi: 10.3389/fncel.2017.00249

Fang, J., Chen, X., Zhang, L., Chen, J., Liang, Y., and Li, X. (2013). P2X7R suppression promotes glioma growth through epidermal growth factor receptor signal pathway. *Int. J. Biochem. Cell Biol.* 45 (6), 1109–1120. doi: 10.1016/j.biocel.2013.03.005

Feng, Y. H., Li, X., Wang, L., Zhou, L., and Gorodeski, G. I. (2006). A truncated P2X7 receptor variant (P2X7-j) endogenously expressed in cervical cancer cells antagonizes the full-length P2X7 receptor through hetero-oligomerization. *J. Biol. Chem.* 281 (25), 17228–17237. doi: 10.1074/jbc.M602999200

Feng, Y. H., Li, X., Zeng, R., and Gorodeski, G. I. (2006). Endogenously expressed truncated P2X7 receptor lacking the C-terminus is preferentially upregulated in epithelial cancer cells and fails to mediate ligand-induced pore formation and apoptosis. *Nucleos. Nucleot. Nucleic Acids* 25 (9-11), 1271–1276. doi: 10.1080/15257770600890921

Ferrari, D., Munerati, M., Melchiorri, L., Hanau, S., di Virgilio, F., and Baricordi, O. R. (1994). Responses to extracellular ATP of lymphoblastoid cell lines from Duchenne muscular dystrophy patients. *Am. J. Physiol.* 267 (4 Pt 1), C886–C892. doi: 10.1152/ajpcell.1994.267.4.C886

Ferrari, D., Chiozzi, P., Falzoni, S., Dal Susino, M., Collo, G., and Buell, G. (1997a). ATP-mediated cytotoxicity in microglial cells. *Neuropharmacology* 36 (9), 1295–1301. doi: 10.1016/s0028-3908(97)00137-8

Ferrari, D., Chiozzi, P., Falzoni, S., Dal Susino, M., Melchiorri, L., and Baricordi, O. R. (1997b). Extracellular ATP triggers IL-1 beta release by activating the purinergic P2Z receptor of human macrophages. *J. Immunol.* 159 (3), 1451–1458.

Ferrari, D., La Sala, A., Chiozzi, P., Morelli, A., Falzoni, S., and Girolomoni, G. (2000). The P2 purinergic receptors of human dendritic cells: identification and coupling to cytokine release. *FASEB J.* 14 (15), 2466–2476. doi: 10.1096/fj.00-0031com

Ferrari, D., Pizzirani, C., Adinolfi, E., Lemoli, R. M., Curti, A., and Idzko, M. (2006). The P2X7 receptor: a key player in IL-1 processing and release. *J. Immunol.* 176 (7), 3877–3883. doi: 10.4049/jimmunol.176.7.3877

Figliuolo, V. R., Savio, L. E. B., Safya, H., Nanini, H., Bernardazzi, C., and Abalo, A. (2017). P2X7 receptor promotes intestinal inflammation in chemically induced colitis and triggers death of mucosal regulatory T cells. *Biochim. Biophys. Acta* 1863 (6), 1183–1194. doi: 10.1016/j.bbadis.2017.03.004

Franceschini, A., Capece, M., Chiozzi, P., Falzoni, S., Sanz, J. M., and Sarti, A. C. (2015). The P2X7 receptor directly interacts with the NLRP3 inflammasome scaffold protein. *FASEB J.* 29 (6), 2450–2461. doi: 10.1096/fj.14-268714

Galluzzi, L., Buque, A., Kepp, O., Zitvogel, L., and Kroemer, G. (2017). Immunogenic cell death in cancer and infectious disease. *Nat. Rev. Immunol.* 17 (2), 97–111. doi: 10.1038/nri.2016.107

Garcia-Marcos, M., Perez-Andres, E., Tandel, S., Fontanils, U., Kumps, A., and Kabre, E. (2006). Coupling of two pools of P2X7 receptors to distinct intracellular signaling pathways in rat submandibular gland. *J. Lipid Res.* 47 (4), 705–714. doi: 10.1194/jlr.M500408-JLR200

Gartland, A., Hipskind, R. A., Gallagher, J. A., and Bowler, W. B. (2001). Expression of a P2X7 receptor by a subpopulation of human osteoblasts. *J. Bone Miner. Res.* 16 (5), 846–856. doi: 10.1359/jbmr.2001.16.5.846

Gartland, A., Skarratt, K. K., Hocking, L. J., Parsons, C., Stokes, L., and Jorgensen, N. R. (2012). Polymorphisms in the P2X7 receptor gene are associated with low lumbar spine bone mineral density and accelerated bone loss in post-menopausal women. *Eur. J. Hum. Genet.* 20 (5), 559–564. doi: 10.1038/ejhg.2011.245

Gavin, M. A., Rasmussen, J. P., Fontenot, J. D., Vasta, V., Manganiello, V. C., and Beavo, J. A. (2007). Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* 445 (7129), 771–775. doi: 10.1038/nature05543

Gehring, M. P., Pereira, T. C., Zanin, R. F., Borges, M. C., Braga Filho, A., and Battastini, A. M. (2012). P2X7 receptor activation leads to increased cell death in a radiosensitive human glioma cell line. *Purinerg. Signal* 8 (4), 729–739. doi: 10.1007/s11302-012-9319-2

Gehring, M. P., Kipper, F., Nicoletti, N. F., Sperotto, N. D., Zanin, R., and Tamajusuku, A. S. (2015). P2X7 receptor as predictor gene for glioma radiosensitivity and median survival. *Int. J. Biochem. Cell Biol.* 68, 92–100. doi: 10.1016/j.biocel.2015.09.001

Ghalali, A., Wiklund, F., Zheng, H., Stenius, U., and Hogberg, J. (2014). Atorvastatin prevents ATP-driven invasiveness via P2X7 and EHBP1 signaling in PTEN-expressing prostate cancer cells. *Carcinogenesis* 35 (7), 1547–1555. doi: 10.1093/carcin/bgu019

Ghiringhelli, F., Apetoh, L., Tesniere, A., Aymeric, L., Ma, Y., and Ortiz, C. (2009). Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. *Nat. Med.* 15 (10), 1170–1178. doi: 10.1038/nm.2028

Giannuzzo, A., Pedersen, S. F., and Novak, I. (2015). The P2X7 receptor regulates cell survival, migration and invasion of pancreatic ductal adenocarcinoma cells. *Mol. Cancer* 14, 203. doi: 10.1186/s12943-015-0472-4

Giannuzzo, A., Saccomano, M., Napp, J., Ellegaard, M., Alves, F., and Novak, I. (2016). Targeting of the P2X7 receptor in pancreatic cancer and stellate cells. *Int. J. Cancer* 139 (11), 2540–2552. doi: 10.1002/ijc.30380

Gidley-Baird, A., and Barden, J. A. (2002). *Antibodies to non-functional p2x7 receptor diagnosis and treatment of cancers and other conditions*.Patent No: WO2003020762A1. Available at: https://patents.google.com/patent/WO2003020762A1/3Den

Gidlof, O., Smith, J. G., Melander, O., Lovkvist, H., Hedblad, B., and Engstrom, G. (2012). A common missense variant in the ATP receptor P2X7 is associated with reduced risk of cardiovascular events. *PloS One* 7 (5), e37491. doi: 10.1371/journal.pone.0037491

Gilbert, S. M., Gidley Baird, A., Glazer, S., Barden, J. A., Glazer, A., and Teh, L. C. (2017). A phase I clinical trial demonstrates that nfP2X7 -targeted antibodies provide a novel, safe and tolerable topical therapy for basal cell carcinoma. *Br. J. Dermatol.* 177 (1), 117–124. doi: 10.1111/bjd.15364

Gilbert, S. M., Oliphant, C. J., Hassan, S., Peille, A. L., Bronsert, P., and Falzoni, S. (2019). ATP in the tumour microenvironment drives expression of nfP2X7, a key mediator of cancer cell survival. *Oncogene* 38 (2), 194–208. doi: 10.1038/s41388-018-0426-6

Giuliani, A. L., Colognesi, D., Ricco, T., Roncato, C., Capece, M., and Amoroso, F. (2014). Trophic activity of human P2X7 receptor isoforms A and B in osteosarcoma. *PloS One* 9 (9), e107224. doi: 10.1371/journal.pone.0107224

Gomez-Villafuertes, R., Garcia-Huerta, P., Diaz-Hernandez, J. I., and Miras-Portugal, M. T. (2015). PI3K/Akt signaling pathway triggers P2X7 receptor expression as a pro-survival factor of neuroblastoma cells under limiting growth conditions. *Sci. Rep.* 5, 18417. doi: 10.1038/srep18417

Gonnord, P., Delarasse, C., Auger, R., Benihoud, K., Prigent, M., and Cuif, M. H. (2009). Palmitoylation of the P2X7 receptor, an ATP-gated channel, controls its expression and association with lipid rafts. *FASEB J.* 23 (3), 795–805. doi: 10.1096/fj.08-114637

Gopalakrishnan, V., Helmink, B. A., Spencer, C. N., Reuben, A., and Wargo, J. A. (2018). The Influence of the Gut Microbiome on Cancer, Immunity, and Cancer Immunotherapy. *Cancer Cell* 33 (4), 570–580. doi: 10.1016/j.ccell.2018.03.015

Graziano, F., Desdouits, M., Garzetti, L., Podini, P., Alfano, M., and Rubartelli, A. (2015). Extracellular ATP induces the rapid release of HIV-1 from virus containing compartments of human macrophages. *Proc. Natl. Acad. Sci. U. S. A* 112 (25), E3265–E3273. doi: 10.1073/pnas.1500656112

Greig, A. V., Linge, C., Healy, V., Lim, P., Clayton, E., and Rustin, M. H. (2003). Expression of purinergic receptors in non-melanoma skin cancers and their functional roles in A431 cells. *J. Invest. Dermatol.* 121 (2), 315–327. doi: 10.1046/j.1523-1747.2003.12379.x

Grol, M. W., Pereverzev, A., Sims, S. M., and Dixon, S. J. (2013). P2 receptor networks regulate signaling duration over a wide dynamic range of ATP concentrations. *J. Cell Sci.* 126 (Pt 16), 3615–3626. doi: 10.1242/jcs.122705

Gu, B. J., and Wiley, J. S. (2006). Rapid ATP-induced release of matrix metalloproteinase 9 is mediated by the P2X7 receptor. *Blood* 107 (12), 4946–4953. doi: 10.1182/blood-2005-07-2994

Gu, B. J., and Wiley, J. S. (2018). P2X7 as a scavenger receptor for innate phagocytosis in the brain. *Br. J. Pharmacol.* 175 (22), 4195–4208. doi: 10.1111/bph.14470

Gu, B., Bendall, L. J., and Wiley, J. S. (1998). Adenosine triphosphate-induced shedding of CD23 and L-selectin (CD62L) from lymphocytes is mediated by the same receptor but different metalloproteases. *Blood* 92 (3), 946–951. doi: 10.1182/blood.V92.3.946

Gu, B. J., Zhang, W. Y., Bendall, L. J., Chessell, I. P., Buell, G. N., and Wiley, J. S. (2000). Expression of P2X(7) purinoceptors on human lymphocytes and monocytes: evidence for nonfunctional P2X(7) receptors. *Am. J. Physiol. Cell Physiol.* 279 (4), C1189–C1197. doi: 10.1152/ajpcell.2000.279.4.C1189

Gu, B. J., Zhang, W., Worthington, R. A., Sluyter, R., Dao-Ung, P., and Petrou, S. (2001). A Glu-496 to Ala polymorphism leads to loss of function of the human P2X7 receptor. *J. Biol. Chem.* 276 (14), 11135–11142. doi: 10.1074/jbc.M010353200

Gu, B. J., Sluyter, R., Skarratt, K. K., Shemon, A. N., Dao-Ung, L. P., and Fuller, S. J. (2004). An Arg307 to Gln polymorphism within the ATP-binding site causes loss of function of the human P2X7 receptor. *J. Biol. Chem.* 279 (30), 31287–31295. doi: 10.1074/jbc.M313902200

Gu, B. J., Rathsam, C., Stokes, L., McGeachie, A. B., and Wiley, J. S. (2009). Extracellular ATP dissociates nonmuscle myosin from P2X(7) complex: this dissociation regulates P2X(7) pore formation. *Am. J. Physiol. Cell Physiol.* 297 (2), C430–C439. doi: 10.1152/ajpcell.00079.2009

Gu, B. J., Saunders, B. M., Jursik, C., and Wiley, J. S. (2010). The P2X7-nonmuscle myosin membrane complex regulates phagocytosis of nonopsonized particles and bacteria by a pathway attenuated by extracellular ATP. *Blood* 115 (8), 1621–1631. doi: 10.1182/blood-2009-11-251744

Gu, L. Q., Li, F. Y., Zhao, L., Liu, Y., Chu, Q., and Zang, X. X. (2010). Association of XIAP and P2X7 receptor expression with lymph node metastasis in papillary thyroid carcinoma. *Endocrine* 38 (2), 276–282. doi: 10.1007/s12020-010-9384-7

Gu, B. J., Saunders, B. M., Petrou, S., and Wiley, J. S. (2011). P2X(7) is a scavenger receptor for apoptotic cells in the absence of its ligand, extracellular ATP. *J. Immunol.* 187 (5), 2365–2375. doi: 10.4049/jimmunol.1101178

Gu, B. J., Duce, J. A., Valova, V. A., Wong, B., Bush, A. I., and Petrou, S. (2012). P2X7 receptor-mediated scavenger activity of mononuclear phagocytes toward non-opsonized particles and apoptotic cells is inhibited by serum glycoproteins but remains active in cerebrospinal fluid. *J. Biol. Chem.* 287 (21), 17318–17330. doi: 10.1074/jbc.M112.340885

Gu, B. J., Field, J., Dutertre, S., Ou, A., Kilpatrick, T. J., and Lechner-Scott, J. (2015). A rare P2X7 variant Arg307Gln with absent pore formation function protects against neuroinflammation in multiple sclerosis. *Hum. Mol. Genet.* 24 (19), 5644–5654. doi: 10.1093/hmg/ddv278

Gudipaty, L., Humphreys, B. D., Buell, G., and Dubyak, G. R. (2001). Regulation of P2X(7) nucleotide receptor function in human monocytes by extracellular ions and receptor density. *Am. J. Physiol. Cell Physiol.* 280 (4), C943–C953. doi: 10.1152/ajpcell.2001.280.4.C943

Guerra, A. N., Gavala, M. L., Chung, H. S., and Bertics, P. J. (2007). Nucleotide receptor signalling and the generation of reactive oxygen species. *Purinerg. Signal* 3 (1-2), 39–51. doi: 10.1007/s11302-006-9035-x

Gulbransen, B. D., Bashashati, M., Hirota, S. A., Gui, X., Roberts, J. A., and MacDonald, J. A. (2012). Activation of neuronal P2X7 receptor-pannexin-1 mediates death of enteric neurons during colitis. *Nat. Med.* 18 (4), 600–604. doi: 10.1038/nm.2679

Gunosewoyo, H., and Kassiou, M. (2010). P2X purinergic receptor ligands: recently patented compounds. *Expert Opin. Ther. Pat.* 20 (5), 625–646. doi: 10.1517/13543771003702424

Guo, C., Masin, M., Qureshi, O. S., and Murrell-Lagnado, R. D. (2007). Evidence for functional P2X4/P2X7 heteromeric receptors. *Mol. Pharmacol.* 72 (6), 1447–1456. doi: 10.1124/mol.107.035980

Gutierrez-Martin, Y., Bustillo, D., Gomez-Villafuertes, R., Sanchez-Nogueiro, J., Torregrosa-Hetland, C., and Binz, T. (2011). P2X7 receptors trigger ATP exocytosis and modify secretory vesicle dynamics in neuroblastoma cells. *J. Biol. Chem.* 286 (13), 11370–11381. doi: 10.1074/jbc.M110.139410

Hale, J. S., Youngblood, B., Latner, D. R., Mohammed, A. U., Ye, L., and Akondy, R. S. (2013). Distinct memory CD4+ T cells with commitment to T follicular helper- and T helper 1-cell lineages are generated after acute viral infection. *Immunity* 38 (4), 805–817. doi: 10.1016/j.immuni.2013.02.020

Han, J., Liu, H., Liu, C., Jin, H., Perlmutter, J. S., and Egan, T. M. (2017). Pharmacologic characterizations of a P2X7 receptor-specific radioligand, [11C] GSK1482160 for neuroinflammatory response. *Nucl. Med. Commun.* 38 (5), 372–382. doi: 10.1097/MNM.0000000000000660

Hansen, M. A., Barden, J. A., Balcar, V. J., Keay, K. A., and Bennett, M. R. (1997). Structural motif and characteristics of the extracellular domain of P2X receptors. *Biochem. Biophys. Res. Commun.* 236 (3), 670–675. doi: 10.1006/bbrc.1997.6815

Harkat, M., Peverini, L., Cerdan, A. H., Dunning, K., Beudez, J., and Martz, A. (2017). On the permeation of large organic cations through the pore of ATP-gated P2X receptors. *Proc. Natl. Acad. Sci. U. S. A* 114 (19), E3786–E3E95. doi: 10.1073/pnas.1701379114

Hattori, M., and Gouaux, E. (2012). Molecular mechanism of ATP binding and ion channel activation in P2X receptors. *Nature* 485 (7397), 207–212. doi: 10.1038/nature11010

Hattori, F., Ohshima, Y., Seki, S., Tsukimoto, M., Sato, M., and Takenouchi, T. (2012). Feasibility study of B16 melanoma therapy using oxidized ATP to target purinergic receptor P2X7. *Eur. J. Pharmacol.* 695 (1-3), 20–26. doi: 10.1016/j.ejphar.2012.09.001

Hibell, A. D., Thompson, K. M., Simon, J., Xing, M., Humphrey, P. P., and Michel, A. D. (2001). Species- and agonist-dependent differences in the deactivation-kinetics of P2X7 receptors. *Naunyn Schmiedebergs Arch. Pharmacol.* 363 (6), 639–648. doi: 10.1007/s002100100412

Hickman, S. E., Khoury, J., Greenberg, S., Schieren, I., and Silverstein, S. C. (1994). P2Z adenosine triphosphate receptor activity in cultured human monocyte-derived macrophages. *Blood* 84 (8), 2452–2456. doi: 10.1182/blood.V84.8.2452.2452

Honore, P., Donnelly-Roberts, D., Namovic, M. T., Hsieh, G., Zhu, C. Z., and Mikusa, J. P. (2006). A-740003 [N-(1-{[(cyanoimino)(5-quinolinylamino) methyl]amino}-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl)acetamide], a novel and selective P2X7 receptor antagonist, dose-dependently reduces neuropathic pain in the rat. *J. Pharmacol. Exp. Ther.* 319 (3), 1376–1385. doi: 10.1124/jpet.106.111559

Honore, P., Donnelly-Roberts, D., Namovic, M., Zhong, C., Wade, C., and Chandran, P. (2009). The antihyperalgesic activity of a selective P2X7 receptor antagonist, A-839977, is lost in IL-1alphabeta knockout mice. *Behav. Brain Res.* 204 (1), 77–81. doi: 10.1016/j.bbr.2009.05.018

Hou, Z., and Cao, J. (2016). Comparative study of the P2X gene family in animals and plants. *Purinerg. Signal* 12 (2), 269–281. doi: 10.1007/s11302-016-9501-z

Hu, J., Ye, F., Cui, M., Lee, P., Wei, C., and Hao, Y. (2016). Protein Profiling of Bladder Urothelial Cell Carcinoma. *PloS One* 11 (9), e0161922. doi: 10.1371/journal.pone.0161922

Huang, S., Chen, Y., Wu, W., Ouyang, N., Chen, J., and Li, H. (2013). miR-150 promotes human breast cancer growth and malignant behavior by targeting the pro-apoptotic purinergic P2X7 receptor. *PloS One* 8 (12), e80707. doi: 10.1371/journal.pone.0080707

Humphreys, B. D., and Dubyak, G. R. (1996). Induction of the P2z/P2X7 nucleotide receptor and associated phospholipase D activity by lipopolysaccharide and IFN-gamma in the human THP-1 monocytic cell line. *J. Immunol.* 157 (12), 5627–5637.

Humphreys, B. D., Rice, J., Kertesy, S. B., and Dubyak, G. R. (2000). Stress-activated protein kinase/JNK activation and apoptotic induction by the macrophage P2X7 nucleotide receptor. *J. Biol. Chem.* 275 (35), 26792–26798. doi: 10.1074/jbc.M002770200

Huo, H., Fryatt, A. G., Farmer, L. K., Schmid, R., and Evans, R. J. (2018). Mapping the binding site of the P2X receptor antagonist PPADS reveals the importance of orthosteric site charge and the cysteine-rich head region. *J. Biol. Chem.* 293 (33), 12820–12831. doi: 10.1074/jbc.RA118.003737

Idzko, M., Dichmann, S., Ferrari, D., Di Virgilio, F., la Sala, A., and Girolomoni, G. (2002). Nucleotides induce chemotaxis and actin polymerization in immature but not mature human dendritic cells via activation of pertussis toxin-sensitive P2y receptors. *Blood* 100 (3), 925–932. doi: 10.1182/blood.v100.3.925

Jacobson, K. A., and Muller, C. E. (2016). Medicinal chemistry of adenosine, P2Y and P2X receptors. *Neuropharmacology* 104, 31–49. doi: 10.1016/j.neuropharm.2015.12.001

Jacobson, K. A., Jarvis, M. F., and Williams, M. (2002). Purine and pyrimidine (P2) receptors as drug targets. *J. Med. Chem.* 45 (19), 4057–4093. doi: 10.1021/jm020046y

Janks, L., Sharma, C. V. R., and Egan, T. M. (2018). A central role for P2X7 receptors in human microglia. *J. Neuroinflammation* 15 (1), 325. doi: 10.1186/s12974-018-1353-8

Jarvis, M. F., and Khakh, B. S. (2009). ATP-gated P2X cation-channels. *Neuropharmacology* 56 (1), 208–215. doi: 10.1016/j.neuropharm.2008.06.067

Jelassi, B., Chantome, A., Alcaraz-Perez, F., Baroja-Mazo, A., Cayuela, M. L., and Pelegrin, P. (2011). P2X(7) receptor activation enhances SK3 channels- and cystein cathepsin-dependent cancer cells invasiveness. *Oncogene* 30 (18), 2108–2122. doi: 10.1038/onc.2010.593

Jelassi, B., Anchelin, M., Chamouton, J., Cayuela, M. L., Clarysse, L., and Li, J. (2013). Anthraquinone emodin inhibits human cancer cell invasiveness by antagonizing P2X7 receptors. *Carcinogenesis* 34 (7), 1487–1496. doi: 10.1093/carcin/bgt099

Ji, Z., Xie, Y., Guan, Y., Zhang, Y., Cho, K. S., and Ji, M. (2018). Involvement of P2X7 Receptor in Proliferation and Migration of Human Glioma Cells. *BioMed. Res. Int.* 2018, 8591397. doi: 10.1155/2018/8591397

Jiang, L. H., Baldwin, J. M., Roger, S., and Baldwin, S. A. (2013). Insights into the Molecular Mechanisms Underlying Mammalian P2X7 Receptor Functions and Contributions in Diseases, Revealed by Structural Modeling and Single Nucleotide Polymorphisms. *Front. Pharmacol.* 4, 55. doi: 10.3389/fphar.2013.00055

Jorgensen, N. R., Husted, L. B., Skarratt, K. K., Stokes, L., Tofteng, C. L., and Kvist, T. (2012). Single-nucleotide polymorphisms in the P2X7 receptor gene are associated with post-menopausal bone loss and vertebral fractures. *Eur. J. Hum. Genet.* 20 (6), 675–681. doi: 10.1038/ejhg.2011.253

Kaczmarek-Hajek, K., Lorinczi, E., Hausmann, R., and Nicke, A. (2012). Molecular and functional properties of P2X receptors–recent progress and persisting challenges. *Purinerg. Signal* 8 (3), 375–417. doi: 10.1007/s11302-012-9314-7

Kaiho, H., Matsuoka, I., Kimura, J., and Nakanishi, H. (1998). Identification of P2X7 (P2Z) receptor in N18TG-2 cells and NG108-15 cells. *J. Neurochem.* 70 (3), 951–957. doi: 10.1046/j.1471-4159.1998.70030951.x

Kan, L. K., Williams, D., Drummond, K., O'Brien, T., and Monif, M. (2019). The role of microglia and P2X7 receptors in gliomas. *J. Neuroimmunol.* 332, 138–146. doi: 10.1016/j.jneuroim.2019.04.010

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

Karasawa, A., and Kawate, T. (2016). Structural basis for subtype-specific inhibition of the P2X7 receptor. *Elife* 5. doi: 10.7554/eLife.22153

Karasawa, A., Michalski, K., Mikhelzon, P., and Kawate, T. (2017). The P2X7 receptor forms a dye-permeable pore independent of its intracellular domain but dependent on membrane lipid composition. *Elife* 6. doi: 10.7554/eLife.31186

Kasuya, G., Fujiwara, Y., Tsukamoto, H., Morinaga, S., Ryu, S., and Touhara, K. (2017). Structural insights into the nucleotide base specificity of P2X receptors. *Sci. Rep.* 7, 45208. doi: 10.1038/srep45208

Kasuya, G., Yamaura, T., Ma, X. B., Nakamura, R., Takemoto, M., and Nagumo, H. (2017). Structural insights into the competitive inhibition of the ATP-gated

P2X receptor channel. *Nat. Commun.* 8 (1), 876. doi: 10.1038/s41467-017-00887-9

Kataoka, A., Tozaki-Saitoh, H., Koga, Y., Tsuda, M., and Inoue, K. (2009). Activation of P2X7 receptors induces CCL3 production in microglial cells through transcription factor NFAT. *J. Neurochem.* 108 (1), 115–125. doi: 10.1111/j.1471-4159.2008.05744.x

Kawate, T., Michel, J. C., Birdsong, W. T., and Gouaux, E. (2009). Crystal structure of the ATP-gated P2X(4) ion channel in the closed state. *Nature* 460 (7255), 592–598. doi: 10.1038/nature08198

Keystone, E. C., Wang, M. M., Layton, M., Hollis, S., McInnes, I. B., and Team, D. C. S. (2012). Clinical evaluation of the efficacy of the P2X7 purinergic receptor antagonist AZD9056 on the signs and symptoms of rheumatoid arthritis in patients with active disease despite treatment with methotrexate or sulphasalazine. *Ann. Rheum. Dis.* 71 (10), 1630–1635. doi: 10.1136/annrheumdis-2011-143578

Khakh, B. S., and Lester, H. A. (1999). Dynamic selectivity filters in ion channels. *Neuron* 23 (4), 653–658. doi: 10.1016/s0896-6273(01)80025-8

Kim, M., Jiang, L. H., Wilson, H. L., North, R. A., and Surprenant, A. (2001). Proteomic and functional evidence for a P2X7 receptor signalling complex. *EMBO J.* 20 (22), 6347–6358. doi: 10.1093/emboj/20.22.6347

Kopp, R., Krautloher, A., Ramirez-Fernandez, A., and Nicke, A. (2019). P2X7 Interactions and Signaling - Making Head or Tail of It. *Front. Mol. Neurosci.* 12, 183. doi: 10.3389/fnmol.2019.00183

Koshimizu, T., Koshimizu, M., and Stojilkovic, S. S. (1999). Contributions of the C-terminal domain to the control of P2X receptor desensitization. *J. Biol. Chem.* 274 (53), 37651–37657. doi: 10.1074/jbc.274.53.37651

Kuehnel, M. P., Rybin, V., Anand, P. K., Anes, E., and Griffiths, G. (2009). Lipids regulate P2X7-receptor-dependent actin assembly by phagosomes via ADP translocation and ATP synthesis in the phagosome lumen. *J. Cell Sci.* 122 (Pt 4), 499–504. doi: 10.1242/jcs.034199

Kunzli, B. M., Berberat, P. O., Giese, T., Csizmadia, E., Kaczmarek, E., and Baker, C. (2007). Upregulation of CD39/NTPDases and P2 receptors in human pancreatic disease. *Am. J. Physiol. Gastrointest. Liver Physiol.* 292 (1), G223–G230. doi: 10.1152/ajpgi.00259.2006

Kunzli, B. M., Bernlochner, M. I., Rath, S., Kaser, S., Csizmadia, E., and Enjyoji, K. (2011). Impact of CD39 and purinergic signalling on the growth and metastasis of colorectal cancer. *Purinerg. Signal* 7 (2), 231–241. doi: 10.1007/s11302-011-9228-9

Kurashima, Y., Amiya, T., Nochi, T., Fujisawa, K., Haraguchi, T., and Iba, H. (2012). Extracellular ATP mediates mast cell-dependent intestinal inflammation through P2X7 purinoceptors. *Nat. Commun.* 3, 1034. doi: 10.1038/ncomms2023

Kwon, J. H., Nam, E. S., Shin, H. S., Cho, S. J., Park, H. R., and Kwon, M. J. (2014). P2X7 Receptor Expression in Coexistence of Papillary Thyroid Carcinoma with Hashimoto's Thyroiditis. *Korean J. Pathol.* 48 (1), 30–35. doi: 10.4132/KoreanJPathol.2014.48.1.30

Lawrence, M. S., Stojanov, P., Mermel, C. H., Robinson, J. T., Garraway, L. A., and Golub, T. R. (2014). Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature* 505 (7484), 495–501. doi: 10.1038/nature12912

Ledderose, C., Woehrle, T., Ledderose, S., Strasser, K., Seist, R., and Bao, Y. (2016). Cutting off the power: inhibition of leukemia cell growth by pausing basal ATP release and P2X receptor signaling? *Purinerg. Signal* 12 (3), 439–451. doi: 10.1007/s11302-016-9510-y

Lenertz, L. Y., Gavala, M. L., Hill, L. M., and Bertics, P. J. (2009). Cell signaling via the P2X(7) nucleotide receptor: linkage to ROS production, gene transcription, and receptor trafficking. *Purinerg. Signal* 5 (2), 175–187. doi: 10.1007/s11302-009-9133-7

Lenertz, L. Y., Wang, Z., Guadarrama, A., Hill, L. M., Gavala, M. L., and Bertics, P. J. (2010). Mutation of putative N-linked glycosylation sites on the human nucleotide receptor P2X7 reveals a key residue important for receptor function. *Biochemistry* 49 (22), 4611–4619. doi: 10.1021/bi902083n

Li, X., Zhou, L., Feng, Y. H., Abdul-Karim, F. W., and Gorodeski, G. I. (2006). The P2X7 receptor: a novel biomarker of uterine epithelial cancers. *Cancer Epidemiol. Biomarkers Prev.* 15 (10), 1906–1913. doi: 10.1158/1055-9965.EPI-06-0407

Li, X., Qi, X., Zhou, L., Catera, D., Rote, N. S., and Potashkin, J. (2007). Decreased expression of P2X7 in endometrial epithelial pre-cancerous and cancer cells. *Gynecol. Oncol.* 106 (1), 233–243. doi: 10.1016/j.ygyno.2007.03.032

Li, X., Qi, X., Zhou, L., Fu, W., Abdul-Karim, F. W., and Maclennan, G. (2009). P2X(7) receptor expression is decreased in epithelial cancer cells of ectodermal, uro-genital sinus, and distal paramesonephric duct origin. *Purinerg. Signal* 5 (3), 351–368. doi: 10.1007/s11302-009-9161-3

Li, M., Toombes, G. E., Silberberg, S. D., and Swartz, K. J. (2015). Physical basis of apparent pore dilation of ATP-activated P2X receptor channels. *Nat. Neurosci.* 18 (11), 1577–1583. doi: 10.1038/nn.4120

Li, X. Y., Moesta, A. K., Xiao, C., Nakamura, K., Casey, M., and Zhang, H. (2019). Targeting CD39 in Cancer Reveals an Extracellular ATP- and Inflammasome-Driven Tumor Immunity. *Cancer Discovery* 9 (12), 1754–1773. doi: 10.1158/2159-8290.CD-19-0541

Liang, X., Samways, D. S. K., Cox, J., and Egan, T. M. (2019). Ca(2+) flux through splice variants of the ATP-gated ionotropic receptor P2X7 is regulated by its cytoplasmic N terminus. *J. Biol. Chem.* 294 (33), 12521–12533. doi: 10.1074/jbc.RA119.009666

Liu, P. S., and Chen, C. Y. (2010). Butyl benzyl phthalate suppresses the ATP-induced cell proliferation in human osteosarcoma HOS cells. *Toxicol. Appl. Pharmacol.* 244 (3), 308–314. doi: 10.1016/j.taap.2010.01.007

Liu, Y., Xiao, Y., and Li, Z. (2011). P2X7 receptor positively regulates MyD88-dependent NF-kappaB activation. *Cytokine* 55 (2), 229–236. doi: 10.1016/j.cyto.2011.05.003

Liu, Z., Liu, Y., Xu, L., An, H., Chang, Y., and Yang, Y. (2015). P2X7 receptor predicts postoperative cancer-specific survival of patients with clear-cell renal cell carcinoma. *Cancer Sci.* 106 (9), 1224–1231. doi: 10.1111/cas.12736

Lopez-Castejon, G., and Brough, D. (2011). Understanding the mechanism of IL-1beta secretion. *Cytokine Growth Factor Rev.* 22 (4), 189–195. doi: 10.1016/j.cytogfr.2011.10.001

Lord, B., Aluisio, L., Shoblock, J. R., Neff, R. A., Varlinskaya, E. I., and Ceusters, M. (2014). Pharmacology of a novel central nervous system-penetrant P2X7 antagonist JNJ-42253432. *J. Pharmacol. Exp. Ther.* 351 (3), 628–641. doi: 10.1124/jpet.114.218487

Lord, B., Ameriks, M. K., Wang, Q., Fourgeaud, L., Vliegen, M., and Verluyten, W. (2015). A novel radioligand for the ATP-gated ion channel P2X7: [3H] JNJ-54232334. *Eur. J. Pharmacol.* 765, 551–559. doi: 10.1016/j.ejphar.2015.09.026

Ma, Y., Adjemian, S., Yang, H., Catani, J. P., Hannani, D., and Martins, I. (2013). ATP-dependent recruitment, survival and differentiation of dendritic cell precursors in the tumor bed after anticancer chemotherapy. *Oncoimmunology* 2 (6), e24568. doi: 10.4161/onci.24568

MacKenzie, A., Wilson, H. L., Kiss-Toth, E., Dower, S. K., North, R. A., and Surprenant, A. (2001). Rapid secretion of interleukin-1beta by microvesicle shedding. *Immunity* 15 (5), 825–835. doi: 10.1016/s1074-7613(01)00229-1

Mackenzie, A. B., Young, M. T., Adinolfi, E., and Surprenant, A. (2005). Pseudoapoptosis induced by brief activation of ATP-gated P2X7 receptors. *J. Biol. Chem.* 280 (40), 33968–33976. doi: 10.1074/jbc.M502705200

Maianski, Z., Pedersen, J., Chabert, C., Barden, J., and S, P. D. (2007). 1435: Evaluation of a New Monoclonal Antibody Targeting the Apoptotic Purinergic Receptor P2x7, as a Diagnostic Tool for Prostate Cancer. *J. Urol.* 177 (4S), 474. doi: 10.1016/S0022-5347(18)31636-7

Mansoor, S. E., Lu, W., Oosterheert, W., Shekhar, M., Tajkhorshid, E., and Gouaux, E. (2016). X-ray structures define human P2X(3) receptor gating cycle and antagonist action. *Nature* 538 (7623), 66–71. doi: 10.1038/nature19367

Martel-Gallegos, G., Casas-Pruneda, G., Ortega-Ortega, F., Sanchez-Armass, S., Olivares-Reyes, J. A., and Diebold, B. (2013). Oxidative stress induced by P2X7 receptor stimulation in murine macrophages is mediated by c-Src/Pyk2 and ERK1/2. *Biochim. Biophys. Acta* 1830 (10), 4650–4659. doi: 10.1016/j.bbagen.2013.05.023

Mawatwal, S., Behura, A., Ghosh, A., Kidwai, S., Mishra, A., and Deep, A. (2017). Calcimycin mediates mycobacterial killing by inducing intracellular calcium-regulated autophagy in a P2RX7 dependent manner. *Biochim. Biophys. Acta Gen. Subj.* 1861 (12), 3190–3200. doi: 10.1016/j.bbagen.2017.09.010

McCarthy, A. E., Yoshioka, C., and Mansoor, S. E. (2019). Full-Length P2X7 Structures Reveal How Palmitoylation Prevents Channel Desensitization. *Cell.* 179 (3), 659–670.e13. doi: 10.1016/j.cell.2019.09.017

McInnes, I. B., Cruwys, S., Bowers, K., and Braddock, M. (2014). Targeting the P2X7 receptor in rheumatoid arthritis: biological rationale for P2X7 antagonism. *Clin. Exp. Rheumatol.* 32 (6), 878–882.

McLarnon, J. G. (2017). Roles of purinergic P2X7 receptor in glioma and microglia in brain tumors. *Cancer Lett.* 402, 93–99. doi: 10.1016/j.canlet.2017.05.004

Mehta, N., Kaur, M., Singh, M., Chand, S., Vyas, B., and Silakari, P. (2014). Purinergic receptor P2X(7): a novel target for anti-inflammatory therapy. *Bioorg. Med. Chem.* 22 (1), 54–88. doi: 10.1016/j.bmc.2013.10.054

Michaud, M., Martins, I., Sukkurwala, A. Q., Adjemian, S., Ma, Y., and Pellegatti, P. (2011). Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. *Science* 334 (6062), 1573–1577. doi: 10.1126/science.1208347

Michel, A. D., and Fonfria, E. (2007). Agonist potency at P2X7 receptors is modulated by structurally diverse lipids. *Br. J. Pharmacol.* 152 (4), 523–537. doi: 10.1038/sj.bjp.0707417

Michel, A. D., Kaur, R., Chessell, I. P., and Humphrey, P. P. (2000). Antagonist effects on human P2X(7) receptor-mediated cellular accumulation of YO-PRO-1. *Br. J. Pharmacol.* 130 (3), 513–520. doi: 10.1038/sj.bjp.0703368

Michel, A. D., Chambers, L. J., and Walter, D. S. (2008a). Negative and positive allosteric modulators of the P2X(7) receptor. *Br. J. Pharmacol.* 153 (4), 737–750. doi: 10.1038/sj.bjp.0707625

Michel, A. D., Clay, W. C., Ng, S. W., Roman, S., Thompson, K., and Condreay, J. P. (2008b). Identification of regions of the P2X(7) receptor that contribute to human and rat species differences in antagonist effects. *Br. J. Pharmacol.* 155 (5), 738–751. doi: 10.1038/bjp.2008.306

Michelsen, K., Yuan, H., and Schwappach, B. (2005). Hide and run. Arginine-based endoplasmic-reticulum-sorting motifs in the assembly of heteromultimeric membrane proteins. *EMBO Rep.* 6 (8), 717–722. doi: 10.1038/sj.embor.7400480

Middaugh, C. R., Mach, H., Burke, C. J., Volkin, D. B., Dabora, J. M., and Tsai, P. K. (1992). Nature of the interaction of growth factors with suramin. *Biochemistry* 31 (37), 9016–9024. doi: 10.1021/bi00152a044

Minkiewicz, J., de Rivero Vaccari, J. P., and Keane, R. W. (2013). Human astrocytes express a novel NLRP2 inflammasome. *Glia* 61 (7), 1113–1121. doi: 10.1002/glia.22499

Mistafa, O., and Stenius, U. (2009). Statins inhibit Akt/PKB signaling via P2X7 receptor in pancreatic cancer cells. *Biochem. Pharmacol.* 78 (9), 1115–1126. doi: 10.1016/j.bcp.2009.06.016

Moncao-Ribeiro, L. C., Faffe, D. S., Santana, P. T., Vieira, F. S., da Graca, C. L., and Marques-da-Silva, C. (2014). P2X7 receptor modulates inflammatory and functional pulmonary changes induced by silica. *PloS One* 9 (10), e110185. doi: 10.1371/journal.pone.0110185

Monif, M., Reid, C. A., Powell, K. L., Smart, M. L., and Williams, D. A. (2009). The P2X7 receptor drives microglial activation and proliferation: a trophic role for P2X7R pore. *J. Neurosci.* 29 (12), 3781–3791. doi: 10.1523/JNEUROSCI.5512-08.2009

Moore, S. F., and MacKenzie, A. B. (2007). Murine macrophage P2X7 receptors support rapid prothrombotic responses. *Cell Signal* 19 (4), 855–866. doi: 10.1016/j.cellsig.2006.10.010

Moore, S. F., and MacKenzie, A. B. (2009). NADPH oxidase NOX2 mediates rapid cellular oxidation following ATP stimulation of endotoxin-primed macrophages. *J. Immunol.* 183 (5), 3302–3308. doi: 10.4049/jimmunol.0900394

Morciano, G., Sarti, A. C., Marchi, S., Missiroli, S., Falzoni, S., and Raffaghello, L. (2017). Use of luciferase probes to measure ATP in living cells and animals. *Nat. Protoc.* 12 (8), 1542–1562. doi: 10.1038/nprot.2017.052

Morelli, A., Chiozzi, P., Chiesa, A., Ferrari, D., Sanz, J. M., and Falzoni, S. (2003). Extracellular ATP causes ROCK I-dependent bleb formation in P2X7-transfected HEK293 cells. *Mol. Biol. Cell* 14 (7), 2655–2664. doi: 10.1091/mbc.02-04-0061

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 (3), 843–857. doi: 10.1038/s41418-019-0474-7

Munoz-Planillo, R., Kuffa, P., Martinez-Colon, G., Smith, B. L., Rajendiran, T. M., and Nunez, G. (2013). K(+) efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* 38 (6), 1142–1153. doi: 10.1016/j.immuni.2013.05.016

Mutini, C., Falzoni, S., Ferrari, D., Chiozzi, P., Morelli, A., and Baricordi, O. R. (1999). Mouse dendritic cells express the P2X7 purinergic receptor: characterization and possible participation in antigen presentation. *J. Immunol.* 163 (4), 1958–1965.

Nelson, D. W., Gregg, R. J., Kort, M. E., Perez-Medrano, A., Voight, E. A., and Wang, Y. (2006). Structure-activity relationship studies on a series of novel, substituted 1-benzyl-5-phenyltetrazole P2X7 antagonists. *J. Med. Chem.* 49 (12), 3659–3666. doi: 10.1021/jm051202e

Noguchi, T., Ishii, K., Fukutomi, H., Naguro, I., Matsuzawa, A., and Takeda, K. (2008). Requirement of reactive oxygen species-dependent activation of ASK1-p38 MAPK pathway for extracellular ATP-induced apoptosis in macrophage. *J. Biol. Chem.* 283 (12), 7657–7665. doi: 10.1074/jbc.M708402200

North, R. A., and Surprenant, A. (2000). Pharmacology of cloned P2X receptors. *Annu. Rev. Pharmacol. Toxicol.* 40, 563–580. doi: 10.1146/annurev.pharmtox.40.1.563

North, R. A. (2002). Molecular physiology of P2X receptors. *Physiol. Rev.* 82 (4), 1013–1067. doi: 10.1152/physrev.00015.2002

Orioli, E., De Marchi, E., Giuliani, A. L., and Adinolfi, E. (2017). P2X7 receptor orchestrates multiple signalling pathways triggering inflammation, autophagy and metabolic/trophic responses. *Curr. Med. Chem.* 24 (21), 2261–2275. doi: 10.2174/0929867324666170303161659

Overes, I. M., de Rijke, B., van Horssen-Zoetbrood, A., Fredrix, H., de Graaf, A. O., and Jansen, J. H. (2008). Expression of P2X5 in lymphoid malignancies results in LRH-1-specific cytotoxic T-cell-mediated lysis. *Br. J. Haematol.* 141 (6), 799–807. doi: 10.1111/j.1365-2141.2008.07125.x

Oyanguren-Desez, O., Rodriguez-Antiguedad, A., Villoslada, P., Domercq, M., Alberdi, E., and Matute, C. (2011). Gain-of-function of P2X7 receptor gene variants in multiple sclerosis. *Cell Calcium* 50 (5), 468–472. doi: 10.1016/j.ceca.2011.08.002

Pandolfi, J. B., Ferraro, A. A., Sananez, I., Gancedo, M. C., Baz, P., and Billordo, L. A. (2016). ATP-Induced Inflammation Drives Tissue-Resident Th17 Cells in Metabolically Unhealthy Obesity. *J. Immunol.* 196 (8), 3287–3296. doi: 10.4049/jimmunol.1502506

Panenka, W., Jijon, H., Herx, L. M., Armstrong, J. N., Feighan, D., and Wei, T. (2001). P2X7-like receptor activation in astrocytes increases chemokine monocyte chemoattractant protein-1 expression via mitogen-activated protein kinase. *J. Neurosci.* 21 (18), 7135–7142. doi: 10.1523/JNEUROSCI.21-18-07135.2001

Panupinthu, N., Rogers, J. T., Zhao, L., Solano-Flores, L. P., Possmayer, F., and Sims, S. M. (2008). P2X7 receptors on osteoblasts couple to production of lysophosphatidic acid: a signaling axis promoting osteogenesis. *J. Cell Biol.* 181 (5), 859–871. doi: 10.1083/jcb.200708037

Park, J. H., and Kim, Y. C. (2017). P2X7 receptor antagonists: a patent review (2010-2015). *Expert Opin. Ther. Pat.* 27 (3), 257–267. doi: 10.1080/13543776.2017.1246538

Park, J. H., Williams, D. R., Lee, J. H., Lee, S. D., Lee, J. H., and Ko, H. (2016). Potent Suppressive Effects of 1-Piperidinylimidazole Based Novel P2X7 Receptor Antagonists on Cancer Cell Migration and Invasion. *J. Med. Chem.* 59 (16), 7410–7430. doi: 10.1021/acs.jmedchem.5b01690

Park, M., Kim, J., Phuong, N. T. T., Park, J. G., Park, J. H., and Kim, Y. C. (2019). Involvement of the P2X7 receptor in the migration and metastasis of tamoxifen-resistant breast cancer: effects on small extracellular vesicles production. *Sci. Rep.* 9 (1), 11587. doi: 10.1038/s41598-019-47734-z

Parvathenani, L. K., Tertyshnikova, S., Greco, C. R., Roberts, S. B., Robertson, B., and Posmantur, R. (2003). P2X7 mediates superoxide production in primary microglia and is up-regulated in a transgenic mouse model of Alzheimer's disease. *J. Biol. Chem.* 278 (15), 13309–13317. doi: 10.1074/jbc.M209478200

Patel, C. H., and Powell, J. D. (2017). Targeting T cell metabolism to regulate T cell activation, differentiation and function in disease. *Curr. Opin. Immunol.* 46, 82–88. doi: 10.1016/j.coi.2017.04.006

Pegoraro, A., Bortolotti, D., Marci, R., Caselli, E., Falzoni, S., and De Marchi, E. (2020). The P2X7 Receptor 489C>T Gain of Function Polymorphism Favors HHV-6A Infection and Associates With Female Idiopathic Infertility. *Front. Pharmacol.* 11 (96). doi: 10.3389/fphar.2020.00096.

Pelegrin, P., Barroso-Gutierrez, C., and Surprenant, A. (2008). P2X7 receptor differentially couples to distinct release pathways for IL-1beta in mouse macrophage. *J. Immunol.* 180 (11), 7147–7157. doi: 10.4049/jimmunol.180.11.7147

Pellegatti, P., Falzoni, S., Pinton, P., Rizzuto, R., and Di Virgilio, F. (2005). A novel recombinant plasma membrane-targeted luciferase reveals a new pathway for

ATP secretion. *Mol. Biol. Cell* 16 (8), 3659–3665. doi: 10.1091/mbc.e05-03-0222

Pellegatti, P., Raffaghello, L., Bianchi, G., Piccardi, F., Pistoia, V., and Di Virgilio, F. (2008). Increased level of extracellular ATP at tumor sites: in vivo imaging with plasma membrane luciferase. *PloS One* 3 (7), e2599. doi: 10.1371/journal.pone.0002599

Peng, L., Bradley, C. J., and Wiley, J. S. (1999). P2Z purinoceptor, a special receptor for apoptosis induced by ATP in human leukemic lymphocytes. *Chin. Med. J. (Engl.)* 112 (4), 356–362.

Perregaux, D. G., and Gabel, C. A. (1998). Human monocyte stimulus-coupled IL-1beta posttranslational processing: modulation via monovalent cations. *Am. J. Physiol.* 275 (6), C1538–C1547. doi: 10.1152/ajpcell.1998.275.6.C1538

Perregaux, D. G., McNiff, P., Laliberte, R., Conklyn, M., and Gabel, C. A. (2000). ATP acts as an agonist to promote stimulus-induced secretion of IL-1 beta and IL-18 in human blood. *J. Immunol.* 165 (8), 4615–4623. doi: 10.4049/jimmunol.165.8.4615

Perruzza, L., Gargari, G., Proietti, M., Fosso, B., D'Erchia, A. M., and Faliti, C. E. (2017). T Follicular Helper Cells Promote a Beneficial Gut Ecosystem for Host Metabolic Homeostasis by Sensing Microbiota-Derived Extracellular ATP. *Cell Rep.* 18 (11), 2566–2575. doi: 10.1016/j.celrep.2017.02.061

Pevarello, P., Bovolenta, S., Tarroni, P., Za, L., Severi, E., and Torino, D. (2017). P2X7 antagonists for CNS indications: recent patent disclosures. *Pharm. Pat. Anal.* 6 (2), 61–76. doi: 10.4155/ppa-2016-0044

Piccioli, P., and Rubartelli, A. (2013). The secretion of IL-1beta and options for release. *Semin. Immunol.* 25 (6), 425–429. doi: 10.1016/j.smim.2013.10.007

Pietrocola, F., Pol, J., Vacchelli, E., Rao, S., Enot, D. P., and Baracco, E. E. (2016). Caloric Restriction Mimetics Enhance Anticancer Immunosurveillance. *Cancer Cell* 30 (1), 147–160. doi: 10.1016/j.ccell.2016.05.016

Pippel, A., Stolz, M., Woltersdorf, R., Kless, A., Schmalzing, G., and Markwardt, F. (2017). Localization of the gate and selectivity filter of the full-length P2X7 receptor. *Proc. Natl. Acad. Sci. U. S. A* 114 (11), E2156–E2E65. doi: 10.1073/pnas.1610414114

Pizzirani, C., Ferrari, D., Chiozzi, P., Adinolfi, E., Sandona, D., and Savaglio, E. (2007). Stimulation of P2 receptors causes release of IL-1beta-loaded microvesicles from human dendritic cells. *Blood* 109 (9), 3856–3864. doi: 10.1182/blood-2005-06-031377

Proietti, M., Perruzza, L., Scribano, D., Pellegrini, G., D'Antuono, R., and Strati, F. (2019). ATP released by intestinal bacteria limits the generation of protective IgA against enteropathogens. *Nat. Commun.* 10 (1), 250. doi: 10.1038/s41467-018-08156-z

Qian, F., Xiao, J., Hu, B., Sun, N., Yin, W., and Zhu, J. (2017). High expression of P2X7R is an independent postoperative indicator of poor prognosis in colorectal cancer. *Hum. Pathol.* 64, 61–68. doi: 10.1016/j.humpath.2017.03.019

Qiu, Y., Li, W. H., Zhang, H. Q., Liu, Y., Tian, X. X., and Fang, W. G. (2014). P2X7 mediates ATP-driven invasiveness in prostate cancer cells. *PloS One* 9 (12), e114371. doi: 10.1371/journal.pone.0114371

Qu, Y., and Dubyak, G. R. (2009). P2X7 receptors regulate multiple types of membrane trafficking responses and non-classical secretion pathways. *Purinerg. Signal* 5 (2), 163–173. doi: 10.1007/s11302-009-9132-8

Qu, Y., Franchi, L., Nunez, G., and Dubyak, G. R. (2007). Nonclassical IL-1 beta secretion stimulated by P2X7 receptors is dependent on inflammasome activation and correlated with exosome release in murine macrophages. *J. Immunol.* 179 (3), 1913–1925. doi: 10.4049/jimmunol.179.3.1913

Raffaghello, L., Chiozzi, P., Falzoni, S., Di Virgilio, F., and Pistoia, V. (2006). The P2X7 receptor sustains the growth of human neuroblastoma cells through a substance P-dependent mechanism. *Cancer Res.* 66 (2), 907–914. doi: 10.1158/0008-5472.CAN-05-3185

Ravenna, L., Sale, P., Di Vito, M., Russo, A., Salvatori, L., and Tafani, M. (2009). Up-regulation of the inflammatory-reparative phenotype in human prostate carcinoma. *Prostate* 69 (11), 1245–1255. doi: 10.1002/pros.20966

Reczek, C. R., and Chandel, N. S. (2015). ROS-dependent signal transduction. *Curr. Opin. Cell Biol.* 33, 8–13. doi: 10.1016/j.ceb.2014.09.010

Reinhold, W. C., Sunshine, M., Liu, H., Varma, S., Kohn, K. W., and Morris, J. (2012). CellMiner: a web-based suite of genomic and pharmacologic tools to explore transcript and drug patterns in the NCI-60 cell line set. *Cancer Res.* 72 (14), 3499–3511. doi: 10.1158/0008-5472.CAN-12-1370

Rizzo, R., Ferrari, D., Melchiorri, L., Stignani, M., Gulinelli, S., and Baricordi, O. R. (2009). Extracellular ATP acting at the P2X7 receptor inhibits secretion of soluble HLA-G from human monocytes. *J. Immunol.* 183 (7), 4302–4311. doi: 10.4049/jimmunol.0804265

Robert, R., Carlile, G. W., Pavel, C., Liu, N., Anjos, S. M., and Liao, J. (2008). Structural analog of sildenafil identified as a novel corrector of the F508del-CFTR trafficking defect. *Mol. Pharmacol.* 73 (2), 478–489. doi: 10.1124/mol.107.040725

Robinson, L. E., and Murrell-Lagnado, R. D. (2013). The trafficking and targeting of P2X receptors. *Front. Cell Neurosci.* 7, 233. doi: 10.3389/fncel.2013.00233

Robinson, L. E., Shridar, M., Smith, P., and Murrell-Lagnado, R. D. (2014). Plasma membrane cholesterol as a regulator of human and rodent P2X7 receptor activation and sensitization. *J. Biol. Chem.* 289 (46), 31983–31994. doi: 10.1074/jbc.M114.574699

Roger, S., Mei, Z. Z., Baldwin, J. M., Dong, L., Bradley, H., and Baldwin, S. A. (2010). Single nucleotide polymorphisms that were identified in affective mood disorders affect ATP-activated P2X7 receptor functions. *J. Psychiatr. Res.* 44 (6), 347–355. doi: 10.1016/j.jpsychires.2009.10.005

Roger, S., Jelassi, B., Couillin, I., Pelegrin, P., Besson, P., and Jiang, L. H. (2015). Understanding the roles of the P2X7 receptor in solid tumour progression and therapeutic perspectives. *Biochim. Biophys. Acta* 1848 (10 Pt B), 2584–2602. doi: 10.1016/j.bbamem.2014.10.029

Rozengurt, E., and Heppel, L. A. (1975). A Specific effect of external ATP on the permeability of transformed 3T3 cells. *Biochem. Biophys. Res. Commun.* 67 (4), 1581–1588. doi: 10.1016/0006-291x(75)90207-7

Ruggeri, R. M., Villari, D., Simone, A., Scarfi, R., Attard, M., and Orlandi, F. (2002). Co-expression of interleukin-6 (IL-6) and interleukin-6 receptor (IL-6R) in thyroid nodules is associated with co-expression of CD30 ligand/CD30 receptor. *J. Endocrinol. Invest.* 25 (11), 959–966. doi: 10.1007/BF03344068

Rump, A., Smolander, O. P., Ruutel Boudinot, S., Kanellopoulos, J. M., and Boudinot, P. (2020). Evolutionary Origin of the P2X7 C-ter Region: Capture of an Ancient Ballast Domain by a P2X4-Like Gene in Ancient Jawed Vertebrates. *Front. Immunol.* 11, 113. doi: 10.3389/fimmu.2020.00113

Ryu, J. K., Jantaratnotai, N., Serrano-Perez, M. C., McGeer, P. L., and McLarnon, J. G. (2011). Block of purinergic P2X7R inhibits tumor growth in a C6 glioma brain tumor animal model. *J. Neuropathol. Exp. Neurol.* 70 (1), 13–22. doi: 10.1097/NEN.0b013e318201d4d4

Salaro, E., Rambaldi, A., Falzoni, S., Amoroso, F. S., Franceschini, A., and Sarti, A. C. (2016). Involvement of the P2X7-NLRP3 axis in leukemic cell proliferation and death. *Sci. Rep.* 6, 26280. doi: 10.1038/srep26280

Salvestrini, V., Orecchioni, S., Talarico, G., Reggiani, F., Mazzetti, C., and Bertolini, F. (2017). Extracellular ATP induces apoptosis through P2X7R activation in acute myeloid leukemia cells but not in normal hematopoietic stem cells. *Oncotarget* 8 (4), 5895–5908. doi: 10.18632/oncotarget.13927

Santos, A. A.Jr., Cappellari, A. R., de Marchi, F. O., Gehring, M. P., Zaparte, A., and Brandao, C. A. (2017). Potential role of P2X7R in esophageal squamous cell carcinoma proliferation. *Purinerg. Signal* 13 (3), 279–292. doi: 10.1007/s11302-017-9559-2

Sanz, J. M., and Di Virgilio, F. (2000). Kinetics and mechanism of ATP-dependent IL-1 beta release from microglial cells. *J. Immunol.* 164 (9), 4893–488. doi: 10.4049/jimmunol.164.9.4893

Savio, L. E. B., de Andrade Mello, P., Figliuolo, V. R., de Avelar Almeida, T. F., Santana, P. T., and Oliveira, S. D. S. (2017). CD39 limits P2X7 receptor inflammatory signaling and attenuates sepsis-induced liver injury. *J. Hepatol.* 67 (4), 716–726. doi: 10.1016/j.jhep.2017.05.021

Schenk, U., Westendorf, A. M., Radaelli, E., Casati, A., Ferro, M., and Fumagalli, M. (2008). Purinergic control of T cell activation by ATP released through pannexin-1 hemichannels. *Sci. Signal* 1 (39), ra6. doi: 10.1126/scisignal.1160583

Schenk, U., Frascoli, M., Proietti, M., Geffers, R., Traggiai, E., and Buer, J. (2011). ATP inhibits the generation and function of regulatory T cells through the activation of purinergic P2X receptors. *Sci. Signal* 4 (162), ra12. doi: 10.1126/scisignal.2001270

Schmid, S., Kubler, M., Korcan Ayata, C., Lazar, Z., Haager, B., and Hossfeld, M. (2015). Altered purinergic signaling in the tumor associated immunologic microenvironment in metastasized non-small-cell lung cancer. *Lung Cancer* 90 (3), 516–521. doi: 10.1016/j.lungcan.2015.10.005

Schrier, S. M., Florea, B. I., Mulder, G. J., Nagelkerke, J. F., and AP, I. J. (2002). Apoptosis induced by extracellular ATP in the mouse neuroblastoma cell line N1E-115: studies on involvement of P2 receptors and adenosine. *Biochem. Pharmacol.* 63 (6), 1119–1126. doi: 10.1016/s0006-2952(01)00939-x

Schwarz, N., Drouot, L., Nicke, A., Fliegert, R., Boyer, O., and Guse, A. H. (2012). Alternative splicing of the N-terminal cytosolic and transmembrane domains of P2X7 controls gating of the ion channel by ADP-ribosylation. *PloS One* 7 (7), e41269. doi: 10.1371/journal.pone.0041269

Shankavaram, U. T., Varma, S., Kane, D., Sunshine, M., Chary, K. K., and Reinhold, W. C. (2009). CellMiner: a relational database and query tool for the NCI-60 cancer cell lines. *BMC Genomics* 10, 277. doi: 10.1186/1471-2164-10-277

Shemon, A. N., Sluyter, R., Fernando, S. L., Clarke, A. L., Dao-Ung, L. P., and Skarratt, K. K. (2006). A Thr357 to Ser polymorphism in homozygous and compound heterozygous subjects causes absent or reduced P2X7 function and impairs ATP-induced mycobacterial killing by macrophages. *J. Biol. Chem.* 281 (4), 2079–2086. doi: 10.1074/jbc.M507816200

Shieh, C. H., Heinrich, A., Serchov, T., van Calker, D., and Biber, K. (2014). P2X7-dependent, but differentially regulated release of IL-6, CCL2, and TNF-alpha in cultured mouse microglia. *Glia* 62 (4), 592–607. doi: 10.1002/glia.22628

Shiratori, M., Tozaki-Saitoh, H., Yoshitake, M., Tsuda, M., and Inoue, K. (2010). P2X7 receptor activation induces CXCL2 production in microglia through NFAT and PKC/MAPK pathways. *J. Neurochem.* 114 (3), 810–819. doi: 10.1111/j.1471-4159.2010.06809.x

Silverman, W. R., de Rivero Vaccari, J. P., Locovei, S., Qiu, F., Carlsson, S. K., and Scemes, E. (2009). The pannexin 1 channel activates the inflammasome in neurons and astrocytes. *J. Biol. Chem.* 284 (27), 18143–18151. doi: 10.1074/jbc.M109.004804

Sinadinos, A., Young, C. N., Al-Khalidi, R., Teti, A., Kalinski, P., and Mohamad, S. (2015). P2RX7 purinoceptor: a therapeutic target for ameliorating the symptoms of duchenne muscular dystrophy. *PloS Med.* 12 (10), e1001888. doi: 10.1371/journal.pmed.1001888

Slater, M., and Barden, J. A. (2005). Differentiating keratoacanthoma from squamous cell carcinoma by the use of apoptotic and cell adhesion markers. *Histopathology* 47 (2), 170–178. doi: 10.1111/j.1365-2559.2005.02155.x

Slater, M., Scolyer, R. A., Gidley-Baird, A., Thompson, J. F., and Barden, J. A. (2003). Increased expression of apoptotic markers in melanoma. *Melanoma Res.* 13 (2), 137–145. doi: 10.1097/01.cmr.0000056225.78713.42

Slater, M., Danieletto, S., Gidley-Baird, A., Teh, L. C., and Barden, J. A. (2004a). Early prostate cancer detected using expression of non-functional cytolytic P2X7 receptors. *Histopathology* 44 (3), 206–15. doi: 10.1111/j.0309-0167.2004.01798.x

Slater, M., Danieletto, S., Pooley, M., Cheng Teh, L., Gidley-Baird, A., and Barden, J. A. (2004b). Differentiation between cancerous and normal hyperplastic lobules in breast lesions. *Breast Cancer Res. Treat* 83 (1), 1–10. doi: 10.1023/B:BREA.0000010670.85915.0f

Slater, M., Danieletto, S., and Barden, J. A. (2005). Expression of the apoptotic calcium channel P2X7 in the glandular epithelium. *J. Mol. Histol.* 36 (3), 159–165. doi: 10.1007/s10735-004-6166-7

Sluyter, R., and Stokes, L. (2011). Significance of P2X7 receptor variants to human health and disease. *Recent Pat. DNA Gene Seq.* 5 (1), 41–54. doi: 10.2174/187221511794839219

Smart, M. L., Gu, B., Panchal, R. G., Wiley, J., Cromer, B., and Williams, D. A. (2003). P2X7 receptor cell surface expression and cytolytic pore formation are regulated by a distal C-terminal region. *J. Biol. Chem.* 278 (10), 8853–8860. doi: 10.1074/jbc.M211094200

Solini, A., Chiozzi, P., Morelli, A., Fellin, R., and Di Virgilio, F. (1999). Human primary fibroblasts in vitro express a purinergic P2X7 receptor coupled to ion fluxes, microvesicle formation and IL-6 release. *J. Cell Sci.* 112 (Pt 3), 297–305.

Solini, A., Cuccato, S., Ferrari, D., Santini, E., Gulinelli, S., and Callegari, M. G. (2008). Increased P2X7 receptor expression and function in thyroid papillary cancer: a new potential marker of the disease? *Endocrinology* 149 (1), 389–396. doi: 10.1210/en.2007-1223

Solini, A., Simeon, V., Derosa, L., Orlandi, P., Rossi, C., and Fontana, A. (2015). Genetic interaction of P2X7 receptor and VEGFR-2 polymorphisms identifies a favorable prognostic profile in prostate cancer patients. *Oncotarget* 6 (30), 28743–28754. doi: 10.18632/oncotarget.4926

Solle, M., Labasi, J., Perregaux, D. G., Stam, E., Petrushova, N., and Koller, B. H. (2001). Altered cytokine production in mice lacking P2X(7) receptors. *J. Biol. Chem.* 276 (1), 125–132. doi: 10.1074/jbc.M006781200

Sorge, R. E., Trang, T., Dorfman, R., Smith, S. B., Beggs, S., and Ritchie, J. (2012). Genetically determined P2X7 receptor pore formation regulates variability in chronic pain sensitivity. *Nat. Med.* 18 (4), 595–599. doi: 10.1038/nm.2710

Steinberg, T. H., Newman, A. S., Swanson, J. A., and Silverstein, S. C. (1987). ATP4- permeabilizes the plasma membrane of mouse macrophages to fluorescent dyes. *J. Biol. Chem.* 262 (18), 8884–8888.

Stock, T. C., Bloom, B. J., Wei, N., Ishaq, S., Park, W., and Wang, X. (2012). Efficacy and safety of CE-224,535, an antagonist of P2X7 receptor, in treatment of patients with rheumatoid arthritis inadequately controlled by methotrexate. *J. Rheumatol.* 39 (4), 720–727. doi: 10.3899/jrheum.110874

Stokes, L., Jiang, L. H., Alcaraz, L., Bent, J., Bowers, K., and Fagura, M. (2006). Characterization of a selective and potent antagonist of human P2X(7) receptors, AZ11645373. *Br. J. Pharmacol.* 149 (7), 880–887. doi: 10.1038/sj.bjp.0706933

Stokes, L., Fuller, S. J., Sluyter, R., Skarratt, K. K., Gu, B. J., and Wiley, J. S. (2010). Two haplotypes of the P2X(7) receptor containing the Ala-348 to Thr polymorphism exhibit a gain-of-function effect and enhanced interleukin-1beta secretion. *FASEB J.* 24 (8), 2916–2927. doi: 10.1096/fj.09-150862

Sun, C., Chu, J., Singh, S., and Salter, R. D. (2010). Identification and characterization of a novel variant of the human P2X(7) receptor resulting in gain of function. *Purinerg. Signal* 6 (1), 31–45. doi: 10.1007/s11302-009-9168-9

Sun, L., Gao, J., Zhao, M., Cui, J., Li, Y., and Yang, X. (2015). A novel cognitive impairment mechanism that astrocytic p-connexin 43 promotes neuronic autophagy via activation of P2X7R and down-regulation of GLT-1 expression in the hippocampus following traumatic brain injury in rats. *Behav. Brain Res.* 291, 315–324. doi: 10.1016/j.bbr.2015.05.049

Sun, S. H. (2010). Roles of P2X7 receptor in glial and neuroblastoma cells: the therapeutic potential of P2X7 receptor antagonists. *Mol. Neurobiol.* 41 (2-3), 351–355. doi: 10.1007/s12035-010-8120-x

Surprenant, A., Rassendren, F., Kawashima, E., North, R. A., and Buell, G. (1996). The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). *Science* 272 (5262), 735–738. doi: 10.1126/science.272.5262.735

Swanson, D. M., Savall, B. M., Coe, K. J., Schoetens, F., Koudriakova, T., and Skaptason, J. (2016). Identification of (R)-(2-Chloro-3-(trifluoromethyl) phenyl)(1-(5-fluoropyridin-2-yl)-4-methyl-6,7-di hydro-1H-imidazo[4,5-c] pyridin-5(4H)-yl)methanone (JNJ 54166060), a Small Molecule Antagonist of the P2X7 receptor. *J. Med. Chem.* 59 (18), 8535–8548. doi: 10.1021/acs.jmedchem.6b00989

Tafani, M., Russo, A., Di Vito, M., Sale, P., Pellegrini, L., and Schito, L. (2010). Up-regulation of pro-inflammatory genes as adaptation to hypoxia in MCF-7 cells and in human mammary invasive carcinoma microenvironment. *Cancer Sci.* 101 (4), 1014–1023. doi: 10.1111/j.1349-7006.2010.01493.x

Tafani, M., Di Vito, M., Frati, A., Pellegrini, L., De Santis, E., and Sette, G. (2011). Pro-inflammatory gene expression in solid glioblastoma microenvironment and in hypoxic stem cells from human glioblastoma. *J. Neuroinflammation* 8, 32. doi: 10.1186/1742-2094-8-32

Takai, E., Tsukimoto, M., Harada, H., Sawada, K., Moriyama, Y., and Kojima, S. (2012). Autocrine regulation of TGF-beta1-induced cell migration by exocytosis of ATP and activation of P2 receptors in human lung cancer cells. *J. Cell Sci.* 125 (Pt 21), 5051–5060. doi: 10.1242/jcs.104976

Takai, E., Tsukimoto, M., Harada, H., and Kojima, S. (2014). Autocrine signaling via release of ATP and activation of P2X7 receptor influences motile activity of human lung cancer cells. *Purinerg. Signal* 10 (3), 487–497. doi: 10.1007/s11302-014-9411-x

Tamajusuku, A. S., Villodre, E. S., Paulus, R., Coutinho-Silva, R., Battasstini, A. M., and Wink, M. R. (2010). Characterization of ATP-induced cell death in the GL261 mouse glioma. *J. Cell Biochem.* 109 (5), 983–991. doi: 10.1002/jcb.22478

Tan, C., Han, L. I., Zou, L., Luo, C., Liu, A., and Sheng, X. (2015). Expression of P2X7R in breast cancer tissue and the induction of apoptosis by the gene-specific shRNA in MCF-7 cells. *Exp. Ther. Med.* 10 (4), 1472–1478. doi: 10.3892/etm.2015.2705

Taylor, S. R., Gonzalez-Begne, M., Dewhurst, S., Chimini, G., Higgins, C. F., and Melvin, J. E. (2008). Sequential shrinkage and swelling underlie P2X7-stimulated lymphocyte phosphatidylserine exposure and death. *J. Immunol.* 180 (1), 300–308. doi: 10.4049/jimmunol.180.1.300

Tsukimoto, M., Tokunaga, A., Harada, H., and Kojima, S. (2009). Blockade of murine T cell activation by antagonists of P2Y6 and P2X7 receptors. *Biochem. Biophys. Res. Commun.* 384 (4), 512–518. doi: 10.1016/j.bbrc.2009.05.011

Ulrich, H., Ratajczak, M. Z., Schneider, G., Adinolfi, E., Orioli, E., and Ferrazoli, E. G. (2018). Kinin and Purine Signaling Contributes to Neuroblastoma Metastasis. *Front. Pharmacol.* 9, 500. doi: 10.3389/fphar.2018.00500

Valera, S., Hussy, N., Evans, R. J., Adami, N., North, R. A., and Surprenant, A. (1994). A new class of ligand-gated ion channel defined by P2x receptor for extracellular ATP. *Nature* 371 (6497), 516–519. doi: 10.1038/371516a0

Vazquez-Cuevas, F. G., Martinez-Ramirez, A. S., Robles-Martinez, L., Garay, E., Garcia-Carranca, A., and Perez-Montiel, D. (2014). Paracrine stimulation of P2X7 receptor by ATP activates a proliferative pathway in ovarian carcinoma cells. *J. Cell Biochem.* 115 (11), 1955–1966. doi: 10.1002/jcb.24867

Vijayan, D., Young, A., Teng, M. W. L., and Smyth, M. J. (2017). Targeting immunosuppressive adenosine in cancer. *Nat. Rev. Cancer* 17 (12), 709–724. doi: 10.1038/nrc.2017.86

Virginio, C., MacKenzie, A., North, R. A., and Surprenant, A. (1999). Kinetics of cell lysis, dye uptake and permeability changes in cells expressing the rat P2X7 receptor. *J. Physiol.* 519 Pt 2, 335–346. doi: 10.1111/j.1469-7793.1999.0335m.x

Vivarelli, S., Salemi, R., Candido, S., Falzone, L., Santagati, M., Stefani, S., et al (2019). Gut Microbiota and Cancer: From Pathogenesis to Therapy. *Cancers (Basel)* 11 (1), 38. doi: 10.3390/cancers11010038

Walev, I., Reske, K., Palmer, M., Valeva, A., and Bhakdi, S. (1995). Potassium-inhibited processing of IL-1 beta in human monocytes. *EMBO J.* 14 (8), 1607–1614. doi: 10.1002/j.1460-2075.1995.tb07149.x

Wang, B., and Sluyter, R. (2013). P2X7 receptor activation induces reactive oxygen species formation in erythroid cells. *Purinerg. Signal* 9 (1), 101–112. doi: 10.1007/s11302-012-9335-2

Wang, L., Jacobsen, S. E., Bengtsson, A., and Erlinge, D. (2004). P2 receptor mRNA expression profiles in human lymphocytes, monocytes and CD34+ stem and progenitor cells. *BMC Immunol.* 5, 16. doi: 10.1186/1471-2172-5-16

Wang, W., Xiao, J., Adachi, M., Liu, Z., and Zhou, J. (2011). 4-aminopyridine induces apoptosis of human acute myeloid leukemia cells via increasing [Ca2+] i through P2X7 receptor pathway. *Cell Physiol. Biochem.* 28 (2), 199–208. doi: 10.1159/000331731

Wei, W., Ryu, J. K., Choi, H. B., and McLarnon, J. G. (2008). Expression and function of the P2X(7) receptor in rat C6 glioma cells. *Cancer Lett.* 260 (1-2), 79–87. doi: 10.1016/j.canlet.2007.10.025

Wesselius, A., Bours, M. J., Arts, I. C., Theunisz, E. H., Geusens, P., and Dagnelie, P. C. (2012). The P2X(7) loss-of-function Glu496Ala polymorphism affects ex vivo cytokine release and protects against the cytotoxic effects of high ATP-levels. *BMC Immunol.* 13, 64. doi: 10.1186/1471-2172-13-64

White, N., Butler, P. E., and Burnstock, G. (2005). Human melanomas express functional P2 X(7) receptors. *Cell Tissue Res.* 321 (3), 411–418. doi: 10.1007/s00441-005-1149-x

White, E., Mehnert, J. M., and Chan, C. S. (2015). Autophagy, Metabolism, and Cancer. *Clin. Cancer Res.* 21 (22), 5037–5046. doi: 10.1158/1078-0432.CCR-15-0490

Wickert, L. E., Blanchette, J. B., Waldschmidt, N. V., Bertics, P. J., Denu, J. M., and Denlinger, L. C. (2013). The C-terminus of human nucleotide receptor P2X7 is critical for receptor oligomerization and N-linked glycosylation. *PloS One* 8 (5), e63789. doi: 10.1371/journal.pone.0063789

Wiley, J. S., Dao-Ung, L. P., Gu, B. J., Sluyter, R., Shemon, A. N., and Li, C. (2002). A loss-of-function polymorphic mutation in the cytolytic P2X7 receptor gene and chronic lymphocytic leukaemia: a molecular study. *Lancet* 359 (9312), 1114–1119. doi: 10.1016/S0140-6736(02)08156-4

Wiley, J. S., Dao-Ung, L. P., Li, C., Shemon, A. N., Gu, B. J., and Smart, M. L. (2003). An Ile-568 to Asn polymorphism prevents normal trafficking and function of the human P2X7 receptor. *J. Biol. Chem.* 278 (19), 17108–17113. doi: 10.1074/jbc.M212759200

Wiley, J. S., Sluyter, R., Gu, B. J., Stokes, L., and Fuller, S. J. (2011). The human P2X7 receptor and its role in innate immunity. *Tissue Antigens* 78 (5), 321–332. doi: 10.1111/j.1399-0039.2011.01780.x

Wilkaniec, A., Gassowska, M., Czapski, G. A., Cieslik, M., Sulkowski, G., and Adamczyk, A. (2017). P2X7 receptor-pannexin 1 interaction mediates extracellular alpha-synuclein-induced ATP release in neuroblastoma SH-SY5Y cells. *Purinerg. Signal.* 13 (3), 347–361. doi: 10.1007/s11302-017-9567-2

Wilson, H. L., Wilson, S. A., Surprenant, A., and North, R. A. (2002). Epithelial membrane proteins induce membrane blebbing and interact with the P2X7 receptor C terminus. *J. Biol. Chem.* 277 (37), 34017–34023. doi: 10.1074/jbc.M205120200

Woehrle, T., Yip, L., Elkhal, A., Sumi, Y., Chen, Y., and Yao, Y. (2010). Pannexin-1 hemichannel-mediated ATP release together with P2X1 and P2X4 receptors regulate T-cell activation at the immune synapse. *Blood* 116 (18), 3475–3484. doi: 10.1182/blood-2010-04-277707

Xia, J., Yu, X., Tang, L., Li, G., and He, T. (2015). P2X7 receptor stimulates breast cancer cell invasion and migration via the AKT pathway. *Oncol. Rep.* 34 (1), 103–110. doi: 10.3892/or.2015.3979

Yan, Z., Khadra, A., Li, S., Tomic, M., Sherman, A., and Stojilkovic, S. S. (2010). Experimental characterization and mathematical modeling of P2X7 receptor channel gating. *J. Neurosci.* 30 (42), 14213–14224. doi: 10.1523/JNEUROSCI.2390-10.2010

Yan, J., Li, X. Y., Roman Aguilera, A., Xiao, C., Jacoberger-Foissac, C., and Nowlan, B. (2020). Control of Metastases via Myeloid CD39 and NK Cell Effector Function. *Cancer Immunol. Res.* 8 (3), 356–367. doi: 10.1158/2326-6066.CIR-19-0749

Yang, Y. C., Chang, T. Y., Chen, T. C., Lin, W. S., Chang, S. C., and Lee, Y. J. (2016). Functional variant of the P2X7 receptor gene is associated with human papillomavirus-16 positive cervical squamous cell carcinoma. *Oncotarget* 7 (50), 82798–82803. doi: 10.18632/oncotarget.12636

Yang, J., Ma, C., and Zhang, M. (2019). High glucose inhibits osteogenic differentiation and proliferation of MC3T3E1 cells by regulating P2X7. *Mol. Med. Rep.* 20 (6), 5084–5090. doi: 10.3892/mmr.2019.10790

Yip, L., Woehrle, T., Corriden, R., Hirsh, M., Chen, Y., and Inoue, Y. (2009). Autocrine regulation of T-cell activation by ATP release and P2X7 receptors. *FASEB J.* 23 (6), 1685–1693. doi: 10.1096/fj.08-126458

Yoon, M. J., Lee, H. J., Kim, J. H., and Kim, D. K. (2006). Extracellular ATP induces apoptotic signaling in human monocyte leukemic cells, HL-60 and F-36P. *Arch. Pharm. Res.* 29 (11), 1032–1041. doi: 10.1007/BF02969288

Young, C. N., Sinadinos, A., Lefebvre, A., Chan, P., Arkle, S., and Vaudry, D. (2015). A novel mechanism of autophagic cell death in dystrophic muscle regulated by P2RX7 receptor large-pore formation and HSP90. *Autophagy* 11 (1), 113–130. doi: 10.4161/15548627.2014.994402

Young, C. N. J., Chira, N., Rog, J., Al-Khalidi, R., Benard, M., and Galas, L. (2018). Sustained activation of P2X7 induces MMP-2-evoked cleavage and functional purinoceptor inhibition. *J. Mol. Cell Biol.* 10 (3), 229–242. doi: 10.1093/jmcb/mjx030

Yu, T., Junger, W. G., Yuan, C., Jin, A., Zhao, Y., and Zheng, X. (2010). Shockwaves increase T-cell proliferation and IL-2 expression through ATP release, P2X7 receptors, and FAK activation. *Am. J. Physiol. Cell Physiol.* 298 (3), C457–C464. doi: 10.1152/ajpcell.00342.2009

Zanovello, P., Bronte, V., Rosato, A., Pizzo, P., and Di Virgilio, F. (1990). Responses of mouse lymphocytes to extracellular ATP. II. Extracellular ATP causes cell type-dependent lysis and DNA fragmentation. *J. Immunol.* 145 (5), 1545–1550.

Zhang, L. Y., Ibbotson, R. E., Orchard, J. A., Gardiner, A. C., Seear, R. V., and Chase, A. J. (2003). P2X7 polymorphism and chronic lymphocytic leukaemia: lack of correlation with incidence, survival and abnormalities of chromosome 12. *Leukemia* 17 (11), 2097–2100. doi: 10.1038/sj.leu.2403125

Zhang, X. J., Zheng, G. G., Ma, X. T., Yang, Y. H., Li, G., and Rao, Q. (2004). Expression of P2X7 in human hematopoietic cell lines and leukemia patients. *Leuk. Res.* 28 (12), 1313–1322. doi: 10.1016/j.leukres.2004.04.001

Zhang, Y., Cheng, H., Li, W., Wu, H., and Yang, Y. (2019a). Highly-expressed P2X7 receptor promotes growth and metastasis of human HOS/MNNG osteosarcoma cells via PI3K/Akt/GSK3beta/beta-catenin and mTOR/HIF1alpha/VEGF signaling. *Int. J. Cancer* 145 (4), 1068–1082. doi: 10.1002/ijc.32207

Zhang, Y., Ding, J., and Wang, L. (2019b). The role of P2X7 receptor in prognosis and metastasis of colorectal cancer. *Adv. Med. Sci.* 64 (2), 388–394. doi: 10.1016/j.advms.2019.05.002

Zhao, Q., Yang, M., Ting, A. T., and Logothetis, D. E. (2007). PIP(2) regulates the ionic current of P2X receptors and P2X(7) receptor-mediated cell death. *Channels (Austin)* 1 (1), 46–55. doi: 10.4161/chan.3914

Zheng, L., Zhang, X., Yang, F., Zhu, J., Zhou, P., and Yu, F. (2014). Regulation of the P2X7R by microRNA-216b in human breast cancer. *Biochem. Biophys. Res. Commun.* 452 (1), 197–204. doi: 10.1016/j.bbrc.2014.07.101

Zitvogel, L., Ma, Y., Raoult, D., Kroemer, G., and Gajewski, T. F. (2018). The microbiome in cancer immunotherapy: Diagnostic tools and therapeutic strategies. *Science* 359 (6382), 1366–1370. doi: 10.1126/science.aar6918

Zumerle, S., Cali, B., Munari, F., Angioni, R., Di Virgilio, F., and Molon, B. (2019). Intercellular Calcium Signaling Induced by ATP Potentiates Macrophage Phagocytosis. *Cell Rep.* 27 (1), 1–10 e4. doi: 10.1016/j.celrep.2019.03.011

Conflict of Interest: RL is an employee of Biosceptre (UK) Ltd.; JB is an employee of Biosceptre international Ltd.,; SM was an employee of Biosceptre (UK) Ltd; FV serves as a member of Biosceptre Scientific Advisory Board. Biosceptre is a company commercializing nfP2X7 targeted therapies. CH and MP have received a CASE Award together with Biosceptre (UK) Limited to support a Doctoral Training Partnership from the UK Research Council [Biotechnology and Biological Sciences Research Council (BBSRC) (Reference No. BB/M009513/1)].

The remaining 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 Lara, Adinolfi, Harwood, Philpott, Barden, Di Virgilio and McNulty. 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 Pharmacology

ORIGINAL RESEARCH
published: 10 June 2020
doi: 10.3389/fphar.2020.00848

K_V11.1 Potassium Channel and the Na^+/H^+ Antiporter NHE1 Modulate Adhesion-Dependent Intracellular pH in Colorectal Cancer Cells

Jessica Iorio[1], *Claudia Duranti*[1], *Tiziano Lottini*[1], *Elena Lastraioli*[1], *Giacomo Bagni*[1], *Andrea Becchetti*[2] *and Annarosa Arcangeli*[1]*

[1] Section of Internal Medicine, Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy, [2] Department of Biotechnology and Biosciences, University of Milano Bicocca, Milano, Italy

OPEN ACCESS

Edited by:
Lin-Hua Jiang,
University of Leeds, United Kingdom

Reviewed by:
Xiaoling Jia,
Beihang University, China
Lucie Brisson,
Croissance et Cancer, France

***Correspondence:**
Annarosa Arcangeli
annarosa.arcangeli@unifi.it

Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *28 February 2020*
Accepted: *22 May 2020*
Published: *10 June 2020*

Citation:
Iorio J, Duranti C, Lottini T, Lastraioli E, Bagni G, Becchetti A and Arcangeli A (2020) K_V11.1 Potassium Channel and the Na^+/H^+ Antiporter NHE1 Modulate Adhesion-Dependent Intracellular pH in Colorectal Cancer Cells. Front. Pharmacol. 11:848. doi: 10.3389/fphar.2020.00848

Increasing evidence indicates that ion channels and transporters cooperate in regulating different aspects of tumor pathophysiology. In cancer cells, H^+/HCO_3^- transporters usually invert the transmembrane pH gradient typically observed in non-neoplastic cells, which is thought to contribute to cancer malignancy. To what extent the pH-regulating transporters are functionally linked to K^+ channels, which are central regulators of cell membrane potential (V_m), is unclear. We thus investigated in colorectal cancer cells the implication of the pH-regulating transporters and K_V11.1 (also known as hERG1) in the pH modifications stimulated by integrin-dependent cell adhesion. Colorectal cancer cell lines (HCT 116 and HT 29) were seeded onto β1 integrin-dependent substrates, collagen I and fibronectin. This led to a transient cytoplasmic alkalinization, which peaked at 90 min of incubation, lasted approximately 180 min, and was inhibited by antibodies blocking the β1 integrin. The effect was sensitive to amiloride (10 μM) and cariporide (5 μM), suggesting that it was mainly caused by the activity of the Na^+/H^+ antiporter NHE1. Blocking K_V11.1 with E4031 shows that channel activity contributed to modulate the β1 integrin-dependent pH_i increase. Interestingly, both NHE1 and K_V11.1 modulated the colorectal cancer cell motility triggered by β1 integrin-dependent adhesion. Finally, the β1 integrin subunit, K_V11.1 and NHE1 co-immunoprecipitated in colorectal cancer cells seeded onto Collagen I, suggesting the formation of a macromolecular complex following integrin-mediated adhesion. We conclude that the interaction between K_V11.1, NHE1, and β1 integrin contributes to regulate colorectal cancer intracellular pH in relation to the tumor microenvironment, suggesting novel pharmacological targets to counteract pro-invasive and, hence, pro-metastatic behavior in colorectal cancer.

Keywords: hERG1, integrins, Collagen I, beta 1 integrin subunit, cariporide, lateral motility

INTRODUCTION

Ion channels and transporters are progressively emerging as pivotal modulators of different aspects of cancer cell behavior (Arcangeli et al., 2009; Lastraioli et al., 2015a). Such pleiotropic effects can be traced back to the regulation of either V_m (an effect mainly exerted by K^+ channels; Huang and Jan, 2014), or of the concentration and intracellular distribution of specific ion species, such as Ca^{2+} (Bose et al., 2015) and H^+ (Gadsby, 2009), or to the direct modulation of intracellular signaling pathways (Arcangeli et al., 2009; Becchetti et al., 2019). However, the molecular interactions between these mechanisms are poorly understood, and a unified picture of the cancer cell pathophysiology is still missing.

One of the K^+ channels most often dysregulated in cancer is $K_V11.1$ (or hERG1), which regulates the resting V_m in excitable cells (Bauer and Schwarz, 2018), as well as in cancers arising from excitable (e.g. neuroblastomas, Crociani et al., 2003) and non-excitable tissues. In particular, $K_V11.1$ modulates intracellular signaling pathways triggered by integrin-mediated adhesion, both in leukemias (Pillozzi and Arcangeli, 2009) and solid cancers such as the colorectal (Crociani et al., 2013), pancreatic (Lastraioli et al., 2015b), gastric (Crociani et al., 2014) and mammary (Becchetti et al., 2017). The underlying mechanism involves the formation of a macromolecular complex between $K_V11.1$ and β1-integrins, which promotes angiogenesis and triggers metastatic spread (Crociani et al., 2013; Becchetti et al., 2017). In pancreatic ductal adenocarcinoma cells, this occurs through the regulation of f-actin dynamics in filopodia (Manoli et al., 2019).

Cancer proliferation and migration are also controlled by intracellular pH, whose regulation is frequently dysregulated in tumors (Webb et al., 2011). Hence, targeting the pH regulating transporters has been suggested as a therapeutic strategy (Persi et al., 2018). Cancer cells generally display a higher activity of the V-type H^+-ATPases expressed on the plasmalemma (Sennoune et al., 2004), the Na^+/H^+ exchanger NHE1 (Stock and Pedersen, 2017), the monocarboxylate transporters (MCTs) (Pinheiro et al., 2012), the Na^+/HCO_3^- cotransporters (NBCs) (Gorbatenko et al., 2014), and the membrane-associated carbonic anhydrases (CAs), such as CA II and CA IX (Mboge et al., 2018). The concerted activity of these proteins generally leads to an inverted transmembrane pH gradient, characterized by alkalization of intracellular pH (pH_i) and extracellular acidosis. This is considered a hallmark of cancer metabolism (Sharma et al., 2015), being associated with increased tumor proliferation, invasion, metastasis, and thus higher aggressiveness and resistance to treatment (McCarty and Whitaker, 2010). Both the pH_i alkalinization and the extracellular acidosis sustained by the higher activity of H^+ transporters in cancer cells promote cell proliferation, escape from apoptosis and metabolic adaptation (Webb et al., 2011). In addition, the inverted pH gradient is involved in the control of cell migration (Webb et al., 2011). An acid extracellular environment favors the formation of invadopodia and activates proteases that degrade the extracellular matrix, hence favoring cancer cell motility and invasiveness (Busco et al., 2010). Conversely, an alkaline pH_i stimulates cell motility by promoting cytoskeleton assembly and focal adhesion remodeling (Srivastava et al., 2008; Frantz et al., 2008), and hence is one of the main hallmarks of metastatic tumors (Stock and Schwab, 2009). In this scenario, a pivotal role is exerted by integrin receptors and by the Na^+/H^+ antiporter NHE1, whose reciprocal regulatory interaction was discovered in the late eighties (Schwartz et al., 1989; Demaurex et al., 1996). In particular, NHE1 is stimulated by cell adhesion, and in turn regulates cell attachment and spreading onto fibronectin (Harguindey et al., 2005; Stock and Schwab, 2006). In migrating cells, the production of a pH_i gradient along the axis of movement accompanies the accumulation of NHE1 at the migrating front, which localizes close to integrins (Grinstein et al., 1993; Plopper et al., 1995; Ludwig et al., 2013). The acidic pericellular environment at the leading edge is thought to increase the pH_e-dependent avidity of integrins, which facilitates cell-matrix interactions and modulates adhesion strength (Lehenkari and Horton, 1999; Eble and Tuckwell, 2003; Stock et al., 2005; Stock et al., 2008). The corresponding local pH_i increase stimulates the focal adhesion dynamics. First, it supports the F-actin severing activity of cofilin (Frantz et al., 2008), which produces free-barbed-end actin in the lamellipodium. Second, it reduces the affinity of talin for actin (Srivastava et al., 2008). It is thus clear that the pH regulating mechanisms are essential determinants of the tumor microenvironment and the cancer cell crosstalk.

Based on the above premises, we investigated whether the pH-regulating transporters are functionally linked to $K_V11.1$ channels, which are strongly dysregulated in cancer cells, and whose activity is tightly related to integrin receptors in modulating cancer cell proliferation and migration (Becchetti et al., 2019). In particular, we studied if pH regulating mechanisms provide a direct link between integrin-mediated hERG1-dependent cell adhesion and the tumor microenvironment. As a model, we used ColoRectal Cancer (CRC) cells, in which knowledge about $K_V11.1$ physiology is particularly extensive.

MATERIALS AND METHODS

Unless otherwise indicated, chemicals and drugs were purchased from Sigma-Aldrich (St. Louis, USA).

Cell Lines and Cell Culture

The human colon carcinoma cell lines HCT 116 and HT 29 were cultured at 37°C and 5% CO_2 in air, in Roswell Park Memorial Institute (RPMI) 1640 Medium, with sodium bicarbonate (2 g/L) and 2mmol/L L-glutamine ("culture medium"), supplemented with 10% fetal bovine serum (FBS) (Euroclone, Italy). In all experiments, cells were starved overnight in culture medium without serum ("no-serum medium") and detached, prior to experiment, with PBS plus 5 mM EDTA.

Coating of Culture Substrates

Extracellular Matrix (ECM) proteins at the final amount per cm2 of surface area shown in brackets: Fibronectin (FN, 5 μg), Collagen-1 [Col-1, 10 μg; produced as reported in Hallowes

et al. (1980)], Vitronectin [VN, 0.5 μg; produced as reported in Yatohgo et al. (1988)]. Coating with Polylysine (PL, 0.1 μg) was taken as a control of integrin-independent adhesion, while seeding onto uncoated dishes was our "no-adhesion" control. FN and VN were diluted in PBS, Col-1 in serum-free media, PL in bi-distilled water and plated to cover the entire growth surface, followed by 1 h incubation at either 37°C (for Col-1) or room temperature (for FN, VN and PL). After the coating procedure, incubation with Bovine Serum Albumin (BSA) for 15 min at 37° C was performed to block all the uncovered plastic sites.

Measurement of pH_i

To determine pH_i, we used 2′,7′-Bis (2-carboxyethyl)-5 (6)-carboxyfluorescein acetoxymethyl esther (BCECF-AM). Cells were starved and detached as described above, and seeded (5 x 10^4 cells/well) in no-serum medium onto uncoated or coated (see above) 96-well plates (clear bottom 96-well plate, polystyrene, TC-treated, clear flat bottom wells, sterile, w/lid, black; Corning, New York, USA). Cells were then incubated at 37°C in 5% CO_2 for different times, in the absence or presence of different drugs (see below). At selected time points, the medium was removed and BCECF-AM (1μM final concentration in loading solution (HBSS 1X plus 0.01% $NaHCO_3$, pH 7)) was added for 30 min at 37°C and 5% CO_2. After incubation, cells were washed twice with loading solution at room temperature. For measurement of initial (time 0) pH_i, cells were detached, kept in suspension in a 1.5 ml tube and incubated in BCECF-AM-containing solution for 30 min at 37°C. Next, they were washed twice with loading solution, poured in a 96-well plate at 5x10^4 cells/well at room temperature, and immediately transferred to the microplate reader. Fluorescence intensity was immediately measured with a microplate reader (Infinite 200 PRO, Tecan, Switzerland) set at the following wavelengths: 440–490nm for excitation and 535 nm for emission. A calibration curve was set up using a high K^+/Nigericin solution (135 mM KCl, 2 mM K_2HPO_4, 20 mM HEPES, 1.2 mM $CaCl_2$ and 0.8 mM $MgSO_4$), in a range of pH from 5.0 to 8.5. All pH values were calculated using 490/440nm fluorescence ratio and applying standard curve and linear equations, as detailed in Grant and Acosta, 1997.

Modulators of β1 Integrin-Mediated Adhesion

The mouse monoclonal anti-β1 integrin antibody BV7 (anti-β1 Ab, kindly gifted by Prof. P. Defilippi, University of Turin, Italy) (Martìn-Padura et al., 1994) was used to block β1-integrins as described in Hofmann et al. (2001). Briefly, cells were seeded onto Col-I in no serum medium, containing anti-β1 Ab (20 μg/ml), for 90 min at 37°C and 5% CO_2. Cells (mostly detached) were collected, and pH_i mesaurement was performed as above described for time zero condition.

Modulators of pH-Regulating Transporters and K_V11.1

We used the following compounds: 100 μM acetazolamide (CA inhibitor; Parkkila et al., 2000), 10 μM amiloride (inhibitor of NHE1 and epithelial Na^+ channel, ENaC; Masereel et al., 2003), 5 μM cariporide (specific NHE1 inhibitor; Hulikova et al., 2013), 30 μM S0859 (NBC inhibitor; Hulikova et al., 2013), and 40 μM E4031 (Tocris, Bristol, UK; K_V11.1 blocker; Masi et al., 2005),

Drugs were added to the cells seeded on uncoated or coated surfaces at different time points. Preliminarily, all compounds were tested for their potential cytotoxic effects at all the used concentrations, by measuring cell viability with the trypan blue test (Pillozzi et al., 2018), at 30, 90 and 180 min of incubation. None of the modulators had any cytotoxic effect (**Table 1**).

Lateral Motility Assay

Lateral motility was determined using 35 mm dishes and drawing 15 horizontal lines and 3 perpendicular lines on the dish bottom to generate a grid system. Plates were coated with Col-I and 5x10^5 cells were seeded and allowed to attach for 90 min. Three wounds were drawn following the 3 horizontal lines. Subsequently, the following treatments were performed with drugs diluted in RPMI medium: control, E4031 40 μM, E4031 40 μM + cariporide 5 μM. Then, the distances between cells were measured at each mark point (where the 3 horizontal lines crossed the 15 vertical lines) using a light microscope. The widths measured at time 0 correspond to the W_0 parameter. These different 45 points were measured again after 90′. Motility Index (MI) was assessed using the following formula: $MI = 1 - W_t/W_0$, where W_t is the width of the wounds after 90′.

Each treatment was performed in triplicate and the experiments were carried out at least 3 times.

Co-Immunoprecipitation Experiments

For (co)-immunoprecipitation experiments cells were seeded and incubated on Col-I. Cells were gently collected by mild scraping and resuspended in ice cold PBS. Protein extraction, quantification and total lysate incubation with protein A/G agarose beads (Santa Cruz Biotechnology, Texas, USA) were performed as previously reported (Becchetti et al., 2017). In particular, the composition of the lysis buffer was the following:

TABLE 1 | Percentage of alive cells after ICT modulators treatment for 30, 90, and 180 min (± s.e.m).

	30′		90′		180′	
	HCT 116	**HT 29**	**HCT 116**	**HT 29**	**HCT 116**	**HT 29**
Acetazolamide	99.01 ± 0.004	99.02 ± 0.004	98.02 ± 0.003	99.01 ± 0.009	97.02 ± 0.004	96.03 ± 0.004
Amiloride	99.02. ± 0.002	98.01 ± 0.008	99.03 ± 0.002	99.01 ± 0.002	98.02 ± 0.002	98.01 ± 0.004
Cariporide	98.01 ± 0.001	98.02 ± 0.001	99.01 ± 0.001	98.01 ± 0.001	98.03 ± 0.015	98.01 ± 0.001
S0859	99.02 ± 0.004	99.01 ± 0.004	98.01 ± 0.003	99.03 ± 0.009	97.01 ± 0.004	96.02 ± 0.004
E4031	99.01 ± 0.005	99.04 ± 0.003	99.01 ± 0.004	99.02 ± 0.005	99.01 ± 0.005	99.03 ± 0.007

NP40 (150 mM), NaCl (150 mM), Tris-HCl pH 8 (50 mM), EDTA pH 8 (5 mM), NaF (10 mM), $Na_4P_2O_7$ (10 mM), Na_3VO_4 (0.4 mM), and protease inhibitor cocktail (cOmplete Mini-Roche, Germany).

The following antibodies were used at the concentration of 5μg per mg of extracted proteins: LEAF Purified anti-human, Clone TS2/16 (BioLegend, California, USA) to immunoprecipitate the β1-integrin; mAb K_V11.1 (MCK Therapeutics, Italy) to immunoprecipitate K_V11.1. After overnight incubation, the immuno-complex was captured by adding 30 μl of protein A/G agarose beads for 2 h at 4°C (with rolling agitation). The agarose beads were washed 3 times in ice-cold wash buffer and 3 times in ice cold PBS followed by addition of 2X Laemli buffer (10 μl) and boiled for 5 min at 95°C. Afterwards, SDS-PAGE was performed. After electrophoresis, proteins were transferred onto PVDF membrane (previously activated) in blotting buffer under cold condition for 1 h at 100 V. The PVDF membrane was then blocked with 5% BSA in T-PBS (0.1% tween) solution for 3h at room temperature to cover the unspecific antibody binding sites on the membrane. SDS-PAGE and antibody incubation were performed as previously described (Becchetti et al., 2017). The following antibodies were used: anti β1-integrin, RM-12, polyclonal rabbit antibody, dilution 1:1,000 (Immunological Science, Italy); anti-Kv11.1, C54 polyclonal rabbit antibody, dilution 1:1,000 (DI.V.A.L TOSCANA S.R.L., Italy); anti-NHE1 polyclonal rabbit antibody, dilution 1:500 (Novus Biologicals, Colorado, USA) and anti-tubulin, monoclonal mouse, dilution 1:500 (Santa Cruz Biotechnology, Texas, USA). The following day the membrane was washed with T-PBS (0.1% tween) (15 min x 3 times) and appropriate secondary antibody: (i) conjugated with peroxidase enzyme was dissolved in 5% BSA in T-PBS (0.1% tween) (dilution 1:10.000) for at least 45 min and washed (15 min x 3 times), revealing was performed using ECL solution in dark room (anti-C54 primary antibody) and (ii) for all other primary antibodies, IRDYe 800 CW (LI-COR Biosciences, Nebraska, USA) was dissolved in 5% BSA in T-PBS (0.1% tween) (dilution 1:20.000) for at least 45 min and washed (15 min x 3 times) before membrane scanning using LI-COR Odyssey Scanner (Biosciences, Nebraska, USA).

Protein Quantification

Data were analyzed with ImageJ and graphs were plotted with OriginPro8. When quantifying variations in K_V11.1-β1 integrin interaction, the signal for co-immunoprecipitated protein was first divided by the signal of the protein used for immunoprecipitation and then normalized to the signal of the corresponding protein in the total lysate.

Statistical Analysis

OriginPro8 was used for analysis. Data groups were tested for normality (Shapiro-Wilk test) and variance homogeneity (Welch test). Statistical significance for two sample analysis was carried out with unpaired t-test. Multiple comparisons were carried out by One-way ANOVA, with post-hoc Bonferroni test. A p value ≤ 0.05 was considered statistically significant.

RESULTS

Cell Adhesion Mediated by β1 Integrin Produces an Early pH_i Alkalinization in HCT 116 and HT 29 CRC Cells

HCT 116 and HT 29 cells were incubated in serum-free medium for different times (0–180 min) onto different ECM substrates: Col-I, FN, and VN. Polylysine or no-coated plastic surfaces were used as integrin-independent or "no-adhesion" controls, respectively. At different time points, pH_i was determined by BCECF-AM. Although with slightly different time courses, both cell lines underwent an early pH_i increase between 0 and 90 min (**Figures 1A, B**). At 90 min of incubation, pH_i was significantly higher in cells seeded onto Col-I and FN, i.e., two substrates recognized by β1 integrins, which are well expressed in both cell lines (**Table 2**). Indeed, treatment with a β1 integrin blocking antibody (BV/, Martìn-Padura et al., 1994) (indicated as "anti-β1 Ab" in **Figure 1**) not only blocked cell adhesion (panel A' and B'), but also prevented the pH_i increase triggered by cell adhesion onto Col-I. In particular, pH_i increased from 6.7 at time 0 (i.e., before seeding) to 7.2 at 90 min in cells seeded either onto Col-I or FN. In contrast, pH_i remained close to the time 0 value (6.74 ±0.006 for HCT 116 and 6.66±0.014 for HT 29) in CRC cells seeded onto Col-I and treated with the anti-β1 Ab (insets to **Figures 1A, B**). The pH_i alkalinization was much smaller in cells seeded onto VN (in agreement with the very low expression of β3-integrins in both cell lines, see **Table 2**), or polylysine or no-coating conditions. Subsequently (i.e., after 90 min of incubation), the pH_i observed in cells seeded on Col-I and FN progressively returned to the control value. At 180 min, cells displayed a pH_i around 7.0, irrespective of growing conditions (**Figures 1A, B**). Such values were maintained for at least 24 h (the complete data set is given in **Table 3**). We conclude that the β1 integrin-mediated adhesion triggers an early and transient pH_i alkalinization from 6.7 to 7.2 in CRC cells.

The pH_i Variations Triggered by β1 Integrin-Dependent Adhesion in CRC Cells Depend on NHE1 Activation and Are Modulated by K_V11.1 Activity

To better determine the mechanism of integrin-dependent pH_i increase, we applied blockers of the different pH-regulating transporters expressed in CRC cells (**Table 2**). In particular, we tested acetazolamide (a wide CA inhibitor), amiloride (an NHE blocker, in particular of NHE1, as well as of ENaC), cariporide (a specific NHE1 inhibitor), and S0859 (an inhibitor of all NBCs). Drugs were used at the concentrations indicated in Materials and Methods, on cells seeded onto Col-I for 90 min, since the beginning of the experiment. Acetazolamide had no effect on pH_i of either cell line, whereas both amiloride and cariporide produced a statistically significant decrease of pH_i which reached values comparable to those detected in cells before seeding (dotted line in **Figure 2**). The same effect was produced by cariporide. The treatment with S0859 produced a reduction of pH_i, although much lower compared to that obtained with amiloride (**Figures 2A, B**).

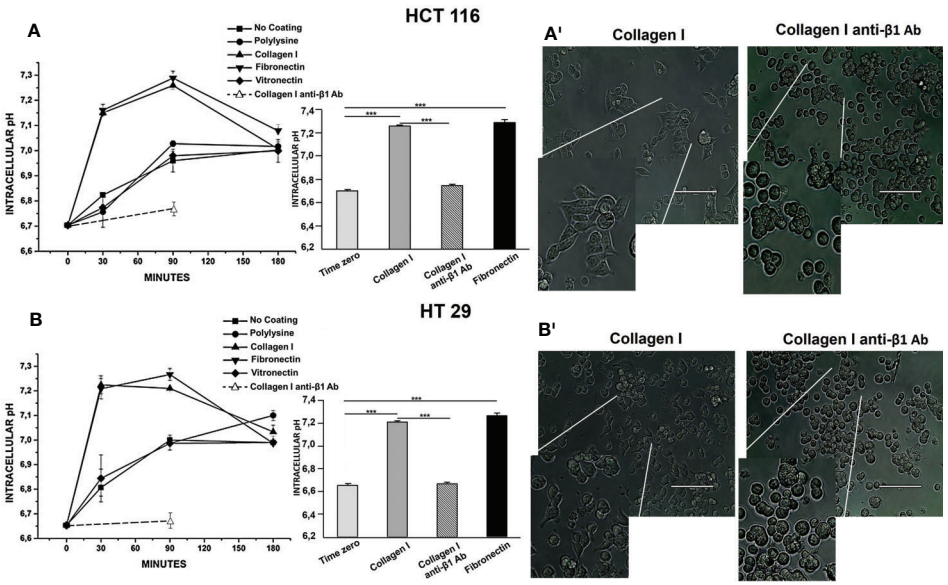


FIGURE 1 | Effect of Collagen I, Fibronectin, and Vitronectin on pH_i in HCT 116 and HT 29 cells. The time course of pH_i is reported in panel **(A)** (HCT 116) and in panel **(B)** (HT 29). Simbol ■: No coating surface, •: PL coating, ▲: Col-I coating, ▼: FN coating, ◆: VN coating, ○: Col-I anti-β1 Ab. On the right of the panel, 90 min pH_i values are reported. Light grey bar: No coating surface, dark grey bar: Col-I, black bar: FN and striped bar: Col-I anti-β1 Ab. Number represent mean ± s.e.m (of three different experiments). *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$. *p value after 30 min of seeding, panel* **(A)**: ****P* < 0.001: Col-I vs Control: 0.0005, FN vs Control: 0.0005; *p value after 90 min of seeding, panel* **(A)**: ****P* < 0.001: Col-I vs Control: 0.0001, FN vs Control: 0.0001; **P* < 0.05: No coat vs Control: 0.01, PL vs Control: 0.01 and VN vs Control: 0.02. *p value after 180 min of seeding, panel* **(A)**: **P* < 0.05: No coat vs Control: 0.01, PL vs Control: 0.01 and VN vs Control: 0.01, Col-I vs Control: 0.01, FN vs Control: 0.01. *p value after 30 min of seeding, panel* **(B)** ****P* < 0.001: Col-I vs Control: 0.0001 and FN vs Control: 0.0001; *p value after 90 min of seeding, panel* **(B)** ****P* < 0.001: Col-I vs Control: 0.0001 and FN vs Control: 0.0001; **P* < 0.05: No coat vs Control: 0.01, PL vs Control: 0.01 and VN vs Control: 0.01. *p value after 180 min of seeding, panel* **(B)** **P* < 0.05: No coat vs Control: 0.01, PL vs Control: 0.01, VN vs Control: 0.01, Col-I vs Control: 0.01 and FN vs Control: 0.01. *p value after 30 min of seeding, panel* **(A)**: ****P* < 0.001: Col-I vs No coat: 0.001 and FN vs No coat: 0.001. *p value after 90 min of seeding, panel* **(A)**: Col-I vs No coat: 0.0001, FN vs No coat: 0.0001; Col-I anti-β1 Ab vs Col-I: 0.0007; Col-I anti-β1 Ab vs FN: 0.0006. *p value after 30 min of seeding, panel* **(B)** ****P* < 0.001: Col-I vs No coat: 0.001 and FN vs No coat: 0.001. *p value after 90 min of seeding, panel* **(B)** Col-I vs No coat: 0.001 and FN vs No coat: 0.001. Col-I anti-β1 Ab vs Col-I: 0.0006; Col-I anti-β1 Ab vs FN: 0.0005. Representative images of cells seeded for 90 min on Col-I, no treated and treated with anti-β1 Ab are reported in panels A^I (HCT 116) and B^I (HT 29), 100 μm scale bar. The conditions are shown on the top of each picture. Cells seeded on Col-I are elongated and attached, cells seeded on Col-I and treated with anti-β1 Ab are round and detached.

TABLE 2 | Integrin profile and ICT expression of HCT 116 and HT 29 cell lines.

	HCT 116	HT 29
$\alpha_1\beta_{1(Col-I)}$, $\alpha_2\beta_{1(Col-I)}$	+ (Boudjadi et al., 2016; Gout et al., 2001)	+ (Boudjadi et al., 2016; Pelillo et al., 2015)
$\alpha_3\beta_{1(FN)}$, $\alpha_4\beta_{1(FN)}$	+ (Pelillo et al., 2015; Ito et al., 2004)	+ (Gout et al., 2001; Ito et al., 2004)
$\alpha_5\beta_{1(FN)}$	+ (Pelillo et al., 2015)	- (Schmidt et al., 1998)
$\alpha_8\beta_{1(FN)}$	- (Benoit et al., 2010)	- (Benoit et al., 2010)
$\alpha_v\beta_{3(FN)}$, $\alpha_v\beta_{3(VN)}$	- (Pelillo et al., 2015)	- (Christenheit et al., 2016)
$\alpha_v\beta_{6(FN)}$	+ (Kim et al., 2018)	+ (Kim et al., 2018)
Kv 11.1	+ (Pillozzi et al., 2018)	+ (Pillozzi et al., 2018)
CA IX	- (McIntyre et al., 2012)	+ (McIntyre et al., 2012)
NHE1	+ (Hulikova et al., 2011)	+ (Hulikova et al., 2011)
NBCe1	+ (Hulikova et al., 2011)	+ (Hulikova et al., 2011)
MCT1	+ (Jin et al., 2019)	+ (Jin et al., 2019)
MCT4	+ (Choi et al., 2019)	+ (Choi et al., 2019)

These results suggest that the early alkalinization triggered by β1 integrin-mediated adhesion is mostly sustained by the activity of the Na^+/H^+ antiporter NHE1, with a lesser contribution of NBC, and scarse involvement of carbonic anhydrases.

We then tested whether $K_V11.1$ was involved in the integrin-dependent pH_i alkalinization. To this purpose, cells were treated with the $K_V11.1$ blocker E4031, at 40 μM (Masi et al., 2005). After 90 min of cell adhesion on Col-I, both HCT 116 and HT 29 cells treated with E4031 showed pH_i values significantly more acidic compared to the untreated controls (**Figures 2A, B**). Hence, the activity of $K_V11.1$ appears to control NHE1 activation, after β1 integrin-mediated adhesion. This

TABLE 3 | Complete set of raw pH_i values.

HCT 116	0'	30'	90'	180'	360'	1440
No Coating	6.70 ± 0.008	6.82 ± 0.005	6.96 ± 0.045	7.00 ± 0.003	7.10 ± 0.004	7.12 ± 0.013
Polylysine	6.70 ± 0.008	6.75 ± 0.036	7.03 ± 0.011	7.02 ± 0.012	7.12 ± 0.013	7.12 ± 0.012
Collagen I	6.70 ± 0.008	7.15 ± 0.009	7.26 ± 0.007	7.01 ± 0.005	7.11 ± 0.008	7.01 ± 0.015
Fibronectin	6.70 ± 0.008	7.16 ± 0.023	7.29 ± 0.026	7.08 ± 0.004	7.05 ± 0.014	7.18 ± 0.04
Vitronectin	6.70 ± 0.008	6.77 ± 0.011	6.98 ± 0.016	6.99 ± 0.045	7.00 ± 0.025	7.10 ± 0.015
HT 29	**0'**	**30'**	**90'**	**180'**	**360'**	**1440**
No Coating	6.65 ± 0.014	6.81±0.035	7.00 ± 0.021	6.99 ± 0.01	7.09 ± 0.01	7.09 ± 0.01
Polylysine	6.65 ± 0.014	6.84 ± 0.096	6.99 ± 0.006	7.12 ± 0.02	7.16 ± 0.001	7.16 ± 0.001
Collagen I	6.65 ± 0.014	7.22 ± 0.037	7.21 ± 0.005	7.03 ± 0.027	7.11 ± 0.023	7.11 ± 0.023
Fibronectin	6.65 ± 0.014	7.21 ± 0.042	7.26 ± 0.024	6.98 ± 0.006	7.03 ± 0.028	7.03 ± 0.028
Vitronectin	6.65 ± 0.014	6.84 ± 0.037	6.98 ± 0.027	6.99 ± 0.011	7.01 ± 0.017	7.01 ± 0.017

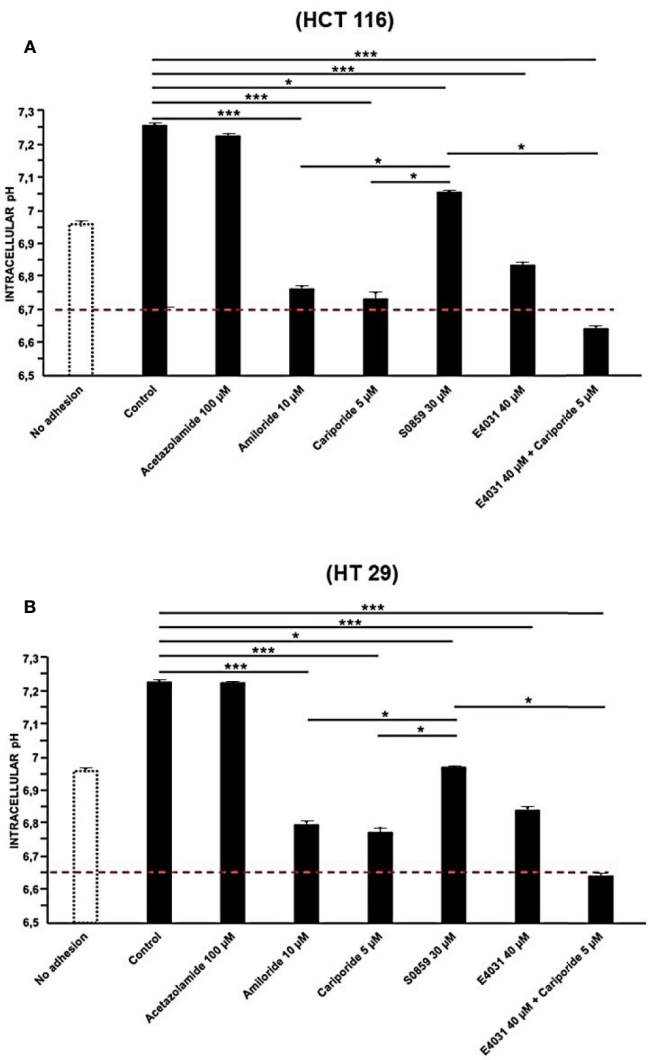


FIGURE 2 | Effect of Acetazolamide, Amiloride, S0859, Cariporide, E4031, and E4031 plus Cariporide on pH_i in cells seeded on collagen I, 90 min treatment, in HCT 116 and HT 29 cells. pH_i values of HCT 116 are reported in panel **(A)** and for HT 29 in panel **(B)**. Red line: pH_i value at time zero. Number represent mean ± s.e.m (of three different experiments). *, $P < 0.05$ and ***, $P < 0.001$. *p value panel* **(A)**: ***P < 0.001: Control vs Amil: $1.9e^{-05}$, Control vs Carip: $1.9e^{-05}$, Control vs E4031: 0.0001, Control vs E4031+Carip: $1.8e^{-05}$Acet vs Carip: $1.7e^{-05}$; *P < 0.05: Control vs S0859: 0.03, Acet vs S0859: 0.03, Amil vs S0859: 0.02; Carip vs S0859: 0.02; S0859 vs E4031+ Carip: 0.02. *p value panel* **(B)**: ***P < 0.001: Control vs Amil: $1.5e^{-05}$, Control vs Carip: $1.4e^{-05}$, Acet vs Carip: $1.3e^{-05}$; Control vs E4031: 0.0001 and Control vs E4031+Carip: $1.9e^{-05}$; *P < 0.05: Control vs S0859: 0.04, Acet vs S0859: 0.03; S0859 vs E4031+ Carip: 0.02.

interpretation was supported by the observation that the combined treatment with E4031 and cariporide had no further effect on the pH_i value obtained after NHE1 inhibition by cariporide (**Figures 2A, B**).

Blockade of Either NHE1 or K_V11.1 Inhibits Lateral Motility of CRC Cells

Next, based on the known correlation between pH_i and cell motility, we performed experiments of lateral motility on our CRC cell lines, which were seeded onto Col-I for 90 min, and treated with either E4031, or cariporide, or a combination of both. Both cariporide and E4031 produced a statistically significant reduction of the motility index compared to untreated cells (**Figures 3A, B**). The combined treatment with E4031 and cariporide only slightly increased the inhibitory effects of the single treatments on the motility index of either cell line. We conclude that both K_V11.1 and NHE1 are involved in controlling the β1 integrin-dependent cell motility in CRC cells.

β1-Integrin, K_V11.1, and NHE1 Form a Macromolecular Complex

We previously showed that cell adhesion onto β1 integrin-dependent substrates (e.g., FN or Col-I), induces K_V11.1 activation, as well as the formation of a macromolecular signaling complex between the channel and β1 integrin on the plasma membrane of HCT 116 cells (Crociani et al., 2013). We thus hypothesized that NHE1 could be also recruited in such complex, which could account for the functional cross-talk between integrin receptors, K_V11.1, and NHE1 in CRC cells. Hence, we seeded HCT116 cells on either uncoated or Col-I-coated surfaces for 90 min, and immunoprecipitated the extracted proteins with anti-β1 integrin or anti-K_V11.1 antibodies. Blots were then revealed, respectively, with anti-K_V11.1 or anti-β1 integrin antibodies, as well as with anti-NHE1 antibodies. We observed that β1-integrin co-immunoprecipitated with both K_V11.1 and NHE1 in CRC cells before cell seeding ("pre seeding" in **Figure 4**), indicating the formation of a β1/Kv11.1/NHE1 complex, whose assembly was further promoted by cell adhesion onto Col-I for 90 min (lanes 3 and 4 in **Figure 4**). On the contrary, in cells seeded onto uncoated surfaces, only a weak co-immunoprecipitation was observed between β1-integrin and K_V11.1, and no association was observed with NHE1. We conclude that cell adhesion onto Col-I stimulates the formation of a macromolecular complex between β1-integrin, K_V11.1, and NHE1.

DISCUSSION

In the present paper, we provide evidence that, in CRC cells, the β1 integrin-mediated adhesion onto ECM proteins such as Col-I and FN triggers an early and transient pH_i alkalinization, from 6.7 to 7.2. The effect is caused by NHE1 activation and is modulated by the activity of the voltage-dependent K^+ channel K_V11.1. The transporter and the channel appear to cooperate in sustaining the ECM-induced CRC cell motility. Their action is accompanied by the formation of a macromolecular complex between the β1 integrin, K_V11.1 and NHE1.

The rapid β1 integrin-dependent pH_i alkalinization in CRC cells is similar to the one initially reported in bovine capillary endothelial cells (Schwartz et al., 1991), which is also induced by integrin engagement (mainly β1), and sustained by activation of the Na^+/H^+ exchanger. In our model, we confirmed the NHE1

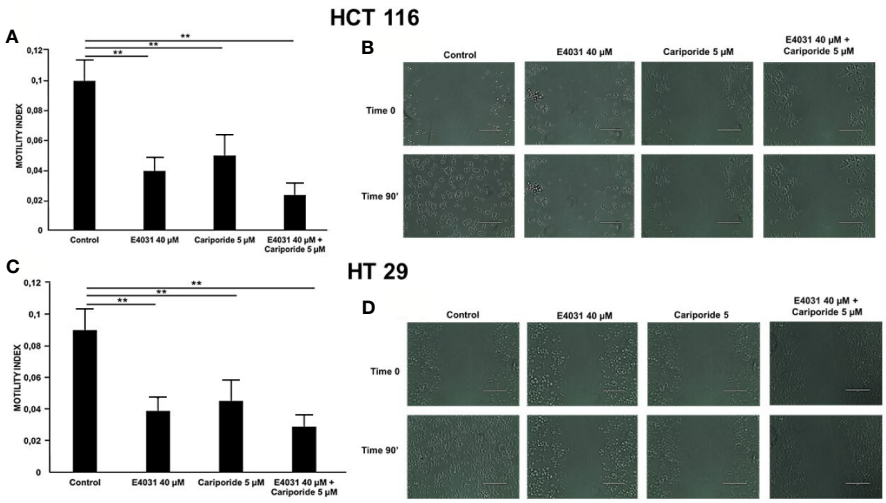


FIGURE 3 | Effect of E4031 and E4031 plus Cariporide on motility index in cells seeded on col-I, 90 min treatment. Motility index values are reported for HCT 116 in panel **(A)** and for HT 29 in panel C. Number represent mean ± s.e.m (of three different experiments). Representative images are reported in panel **(B)** for HCT 116 and in panel **(D)** for HT 29, 200 µm scale bar. In figure the statistically significant differences between control and treatments are reported. **, $P < 0.01$. *p value panel* **(A)**: **, $P < 0.01$: Control vs E4031: 0.002 and Control vs E4031+Carip: 0.001. *p value panel* **(C)**: **, $P < 0.01$: Control vs E4031: 0.003 and Control vs E4031 +Carip: 0.001.

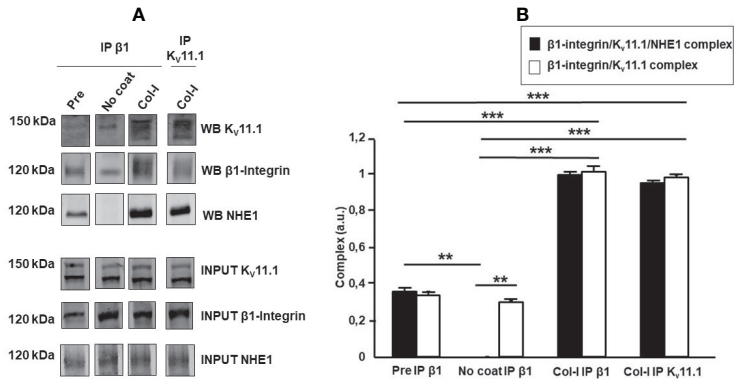


FIGURE 4 | β1-integrin, K_V11.1, and NHE1 protein complex. **(A)** Co-immunoprecipitation of β1 Integrin, Kv 11.1 and NHE1 in HCT 116 cells, seeded on no coating surface and Col-I for 90 min. Densitometric analysis is reported in panel **(A)**. In panel A with "WB" is indicated the protein signal in the co-ip and with "INPUT" the protein signal in the total lysate. Pre-seeding condition is reported as pre, No coating as No coat and Collagen I as Col-I; The immunoprecipitation with anti β1 integrin antibody is indicated as IP β1 and with anti K_V11.1 antibody is reported as IP K_V11.1. Complex quantification is reported in panel B, black bar: β1-integrin, K_V11.1 and NHE1 protein complex and white bar: β1-integrin and K_V11.1 protein complex. Number represent mean ± s.e.m (of three different experiments). **, $P <$ 0.01 and ***, $P < 0.001$. *p value panel* **(B)**, ***, $P < 0.001$: β1-integrin/K_V11.1/NHE1 complex, Pre vs Col-I IP β1, p: 0.0008; β1-integrin/K_V11.1 complex, Pre vs Col-I IP β1, p: 0.00075; β1-integrin/K_V11.1/NHE1 complex, Pre vs Col-I IP K_V11.1, p: 0.00076; β1-integrin/K_V11.1 complex, Pre vs Col-I IP K_V11.1 p: 0.00074. β1-integrin/K_V11.1/NHE1 complex, No coat vs Col-I IP β1, p: $1.7e^{\wedge-05}$; β1-integrin/K_V11.1 complex No coat vs Col-I IP β1, p: 0.00072; β1-integrin/K_V11.1/NHE1 complex, No coat vs Col-I IP K_V11.1, p: $1.9e^{\wedge-05}$; β1-integrin/K_V11.1 complex, No coat vs Col-I IP K_V11.1, p: 0.00073. **, $P < 0.01$: β1-integrin/K_V11.1/NHE1 complex, Pre vs No coat: 0.002; β1-integrin/K_V11.1/NHE1 complex vs β1-integrin/K_V11.1 complex, No coat IP β1: 0.002. Cropped images of blots are reported.

involvement by showing that ECM-dependent alkalinization was blocked by cariporide.

Following Schwartz's seminal observation, the pH regulatory role of NHE1 in normal and cancer cells has been receiving increasing attention (Stock and Pedersen, 2017). In particular, in CRC cells, both H^+ extrusion through NHE1 and HCO_3^- influx through NBCe1 give a significant contribution to pH_i regulation. However, while HCO_3^- influx appears to represent a constitutive element of pH_i regulation, the NHE1-mediated H^+ efflux may vary, depending on culture conditions, e.g. 2D vs 3D cultures (Hulikova et al., 2011). In CRC cells, we found that the Na^+/HCO_3^- cotransporter, although present, provides only a weak contribution to the integrin-dependent alkalinization in CRC cells. In fact, NHE1 appears to constitute the main molecular device linking the ECM microenvironment to pH_i regulation.

Numerous mechanisms leading to NHE1 activation have been described in the various cell types in which the transporter is expressed (Orlowski and Grinstein, 2004). Stimuli such as growth factors, peptide hormones etc., which activate receptor tyrosine kinases and G protein-coupled receptors, enhance NHE1 activity, through the involvement of the mitogen-activated, extracellular signal-related kinase (MEK-ERK)-p90rsk. The latter phosphorylates NHE1, and enables its binding to the multifunctional scaffolding protein 14-3-3, which in turn serves as a hub for the assembly of other signaling molecules which eventually enhance cation exchange (Orlowski and Grinstein, 2004). In this scenario, it is not surprising that integrins exert a stimulatory role on NHE1, as they are known to trigger intracellular signaling pathways that can lead to NHE1 activation (Putney et al., 2002). Integrins, and the tumor microenviroment as a whole, can contribute to trigger a complex signaling pathway which in turn regulates NHE1-dependent motility and invasion in different cancer cells (Cardone et al., 2005). In particular, NHE1 is linked to the actin cytoskeleton and integrates phosphorylation signals arising from kinases which are involved in cytoskeletal reorganization and cell motility. In addition, NHE1 is preferentially localized in pseudopodia, focal adhesion plates, and invadopodia in migrating cells (Paradiso et al., 2004; Patel and Barber, 2005; Clement et al., 2013). In this context, a slight alkalinization mediated by NHE1 was found to regulate the cofilin-mediated actin assembly (Frantz et al., 2008), a central mechanism in cell protrusion. Hence, NHE1 and cofilin respectively act as a pH regulator and a pH sensor, to mediate actin filament assembly.

The most novel result emerging from our data is that the K_V11.1 channel is implicated in the pH_i alkalinization triggered by integrin-mediated cell adhesion to ECM proteins, and sustained by NHE1 activity. K_V11.1 is over-expressed in many cancer types, including CRC (Lastraioli et al., 2004; Lastraioli et al., 2012; Crociani et al., 2013; Iorio et al., 2020). In cancers, β1 integrin-mediated adhesion to FN or Col-I activates K_V11.1, and induces the formation of a macromolecular functional complex on the plasma membrane which comprises the channel and the integrin itself. This occurs preferentially when the channel is in the closed conformation, and leads to the activation of signaling pathways, which also involve the scaffold protein 14-3-3, and in turn control different aspects of cancer cell behavior (Becchetti et al., 2017; Becchetti et al., 2019). The recruitment of NHE1 in the K_V11.1/β1 integrin complex could give rise to the formation of a signaling hub, facilitating NHE1 activation and hence a

localized pH_i alkalinization, which in turn could affect the reorganization of actin filaments, presumably regulated by cofilin activation. This agrees with our recent observations in pancreatic ductal adenocarcinoma cells, where $K_V11.1$ regulates cell migration through a reorganization of f-actin in stress fibers and a modulation of filopodia formation and dynamics (Manoli et al., 2019).

The identification of the signaling mechanisms underlying $K_V11.1$ and NHE1 interaction triggered by β1 integrin–mediated adhesion needs further experiments. Nevertheless, the interplay between a K+ channel and the pH regulating transporter NHE1 that we describe in the present paper can be considered of relevance in the context of CRC invasiveness/motility. This aspect is often dependent on a complex interaction between cancer cells and the tumor microenvironment, and in particular with ECM proteins like collagens and fibronectin (Arcangeli, 2011). Finally, targeting the integrin/ion channel/NHE1 molecular hub might represent a therapeutic option to fight cancer invasiveness.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

JI performed the experiments, analyzed the data, prepared the figures, wrote the manuscript. CD performed the experiments and wrote the manuscript. TL performed the experiments. EL helped in the analysis of the data and reviewed the manuscript. GB contributed to experiments and figures. AB reviewed manuscript. AA designed this project and wrote the manuscript.

FUNDING

This work was supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC, Grant N° IG 21510 and Grant N°IG 15627), by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 813834, by PRIN 2017 (LIONESS) and by Doctorate Course in Genetics, Oncology and Clinical Medicine (Department of Medical Biotechnologies, University of Siena, Siena, Italy).

ACKNOWLEDGMENTS

The authors thank Dr. Massimo D'Amico and Dr. Giulia Petroni for helping with pilot experiments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00848/full#supplementary-material

REFERENCES

Arcangeli, A., Crociani, O., Lastraioli, E., Masi, A., Pillozzi, S., and Becchetti, A. (2009). Targeting ion channels in cancer: a novel frontier in antineoplastic therapy. *Curr. Med. Chem.* 16, 66–93. doi: 10.2174/092986709787002835

Arcangeli, A. (2011). Ion channels and transporters in cancer. 3. Ion channels in the tumor cell-microenvironment cross talk. *Am. J. Physiol. Cell Physiol.* 301, C762–C771. doi: 10.1152/ajpcell.00113.2011

Bauer, C. K., and Schwarz, J. R. (2018). *Ether-à-go-go* K^+ channels: effective modulators of neuronal excitability. *J. Physiol.* 596, 769–783. doi: 10.1113/JP275477

Becchetti, A., Crescioli, S., Zanieri, F., Petroni, G., Mercatelli, R., Coppola, S., et al. (2017). The conformational state of hERG1 channels determines integrin association, downstream signaling, and cancer progression. *Sci. Signal.* 10, eaaf3236. doi: 10.1126/scisignal.aaf3236

Becchetti, A., Petroni, G., and Arcangeli, A. (2019). Ion Channel Conformations Regulate Integrin- Dependent Signaling. *Trends Cell Biol.* 4, 298–307. doi: 10.1016/j.tcb.2018.12.005

Benoit, Y. D., Larrivée, J. F., Groulx, J. F., Stankova, J., Vachon, P. H., and Beaulieu, J. F. (2010). Integrin α8β1 confers anoikis susceptibility to human intestinal epithelial crypt cells. *Biochem. Biophys. Res. Commun.* 399, 434–439. doi: 10.1016/j.bbrc.2010.07.107

Bose, T., Cieślar-Pobuda, A., and Wiechec, E. (2015). Role of ion channels in regulating Ca^{2+} homeostasis during the interplay between immune and cancer cells. *Cell Death Dis.* 6, e1648. doi: 10.1038/cddis.2015.23

Boudjadi, S., Carrier, J., Groulx, J., and Beaulieu, J. F. (2016). Integrin α1β1 expression is controlled by c-MYC in colorectal cancer cells. *Oncogene* 35, 1671– 1678. doi: 10.1038/onc.2015.231

Busco, G., Cardone, R. A., Greco, M. R., Bellizzi, A., Colella, M., Antelmi, E., et al. (2010). NHE1 promotes invadopodial ECM proteolysis through acidification of the periinvadopodial space. *FASEB J.* 24, 3903–3915. doi: 10.1096/fj.09-149518

Cardone, R. A., Casavola, V., and Reshkin, S. J. (2005). The role of disturbed pH dynamics and the Na+/H+ exchanger in metastasis. *Nat. Rev. Cancer* 5, 786–795. doi: 10.1038/nrc1713

Choi, S. H., Kim, M. Y., Yoon, Y. S., Koh, D. I., Kim, M. K., Cho, S. Y., et al. (2019). Hypoxia-induced RelA/p65 derepresses SLC16A3 (MCT4) by downregulating ZBTB7A. *Biochim. Biophys. Acta Gene Regul. Mech.* 1862, 771–785. doi: 10.1016/j.bbagrm.2019.06.004

Christenheit, A., Heffeter, P., and Selzer, E. (2016). A Novel Small-Molecule Integrin Antagonist Inhibits Cells Adhesion Followed By Anoikis in Endothelial Cells - A Comparative Analysis with Cilengitide. *Global J. Cancer Ther.* 2, 009–018. doi: 10.17352/XXXX-XXXX.000008

Clement, D. L., Mally, S., Stock, C., Lethan, M., Satir, P., Schwab, A., et al. (2013). PDGFRα signaling in the primary cilium regulates NHE1-dependent fibroblast migration via coordinated differential activity of MEK1/2-ERK1/2-p90RSK and AKT signaling pathways. *J. Cell Sci.* 126, 953–965. doi: 10.1242/jcs.116426

Crociani, O., Guasti, L., Balzi, M., Becchetti, A., Wanke, E., Olivotto, M., et al. (2003). Cell cycle-dependent expression of HERG1 and HERG1B isoforms in tumor cells. *J. Biol. Chem.* 278, 2947–2955. doi: 10.1074/jbc.M210789200

Crociani, O., Zanieri, F., Pillozzi, S., Lastraioli, E., Stefanini, M., Fiore, A., et al. (2013). hERG1 channels modulate integrin signaling to trigger angiogenesis and tumor progression in colorectal cancer. *Sci. Rep.* 3, 3308. doi: 10.1038/srep03308

Crociani, O., Lastraioli, E., Boni, L., Pillozzi, S., Romoli, M. R., D'Amico, M., et al. (2014). hERG1 channels regulate VEGF-A secretion in human gastric cancer:

clinicopathological correlations and therapeutical implications. *Clin. Cancer Res.* 20, 1502–1512. doi: 10.1158/1078-0432.CCR-13-2633

Demaurex, N., Downey, G. P., Waddell, T. K., and Grinstein, S. (1996). Intracellular pH regulation during spreading of human neutrophils. *J. Cell Biol.* 133, 1391–1402. doi: 10.1083/jcb.133.6.1391

Eble, J. A., and Tuckwell, D. S. (2003). The alpha2beta1 integrin inhibitor rhodocetin binds to the A-domain of the integrin alpha2 subunit proximal to the collagen-binding site. *Biochem. J.* 376, 77–85. doi: 10.1042/bj20030373

Frantz, C., Barreiro, G., Dominguez, L., Chen, X., Eddy, R., Condeelis, J., et al. (2008). Cofilin is a pH sensor for actin free barbed end formation: role of phosphoinositide binding J. *Cell Biol.* 183, 865–879. doi: 10.1083/jcb.200804161

Gadsby, D. C. (2009). Ion channels versus ion pumps: the principal difference, in principle. *Nat. Rev. Mol. Cell Biol.* 10, 344–352. doi: 10.1038/nrm2668

Gorbatenko, A., Olesen, C. W., Boedtkjer, E., and Pedersen, S. F. (2014). Regulation and roles of bicarbonate transporters in cancer. *Front. Physiol.* 5, 130. doi: 10.3389/fphys.2014.00130

Gout, S. P., Jacquier-Sarlin, M. R., Rouard-Talbot, L., Rousselle, P., and Block, M. R. (2001). RhoA-dependent switch between alpha2beta1 and alpha3beta1 integrins is induced by laminin-5 during early stage of HT-29 cell differentiation. *Mol. Biol. Cell.* 12, 3268–3281. doi: 10.1091/mbc.12.10.3268

Grant, R. L., and Acosta, D. (1997). Ratiometric measurement of intracellular pH of cultured cells with BCECF in a fluorescence multi-well plate reader. *Vitro Cell Dev. Biol. Anim.* 33, 256. doi: 10.1007/s11626-997-0044-z

Grinstein, S., Woodside, M., Waddell, T. K., Downey, G. P., J Orlowski, J., Pouyssegur, J., et al. (1993). Focal localization of the NHE-1 isoform of the Na+/H+ antiport: assessment of effects on intracellular pH. *EMBO J.* 1993. 12, 5209–5218. doi: 10.1002/j.1460-2075.1993.tb06216.x

Hallowes, R. C., Bone, E. J., and Jones, W. (1980). A new dimension in the culture in the human breast, in Tissue culture in Medical research, vol. 2. Eds. R. J. Richards and K. T. Rajan (Pergamon Oxford), 215–244.

Harguindey, S., Orive, G., Luis Pedraz, J., Paradiso, A., and Reshkin, S. J. (2005). The role of pH dynamics and the Na+/H+antiporter in the etiopathogenesis and treatment of cancer. Two faces of the some coin — one single nature. *Biochim. Biophys. Acta* 1756, 1–24. doi: 10.1016/j.bbcan.2005.06.004

Hofmann, G., Bernabei, P. A., Crociani, O., Cherubini, A., Guasti, L., Pillozzi, S., et al. (2001). HERG K^+ Channels Activation during β_1Integrin-mediated Adhesion to Fibronectin Induces an Up-regulation of $\alpha_v\beta_3$ Integrin in the Preosteoclastic Leukemia Cell Line FLG 29.1. *JBC* 276, 4923–4931. doi: 10.1074/jbc.M005682200

Huang, X., and Jan, L. Y. (2014). Targeting potassium channels in cancer. *J. Cell Biol.* 206, 151–162. doi: 10.1083/jcb.201404136

Hulikova, A., Vaughan-Jones, R. D., and Swietach, P. (2011). Dual role of CO_2/HCO_3^- buffer in the regulation of intracellular pH of three-dimensional tumor growths. *J. Biol. Chem.* 286, 13815– 13826. doi: 10.1074/jbc.M111.219899

Hulikova, A., Harris, A. L., Vaughan-Jones, R. D., and Swietach, P. (2013). Regulation of intracellular pH in cancer cell lines under normoxia and hypoxia. *J. Cell Physiol.* 228, 743–752. doi: 10.1002/jcp.24221

Iorio, J., Lastraioli, E., Tofani, L., Petroni, G., Antonuzzo, L., Messerini, L., et al. (2020). hERG1 and HIF-2α Behave as Biomarkers of Positive Response to Bevacizumab in Metastatic Colorectal Cancer Patients. *Transl. Oncol.* 13, 100740. doi: 10.1016/j.tranon.2020.01.001

Ito, H., Rocha, F. G., Duxbury, M., Zinner, M. J., Ashley, S. W., and Whang, E. E. (2004). Fibronectin induces colon cancer MMP-2 activation through a Src- and NfκB-dependent mechanism. *J. Surg. Res.* 121, 299. doi: 10.1016/j.jss.2004.07.106

Jin, P., Jiang, J., Xie, N., Zhou, L., Huang, Z., Zhang, L., et al. (2019). MCT1 relieves osimertinib-induced CRC suppression by promoting autophagy through the LKB1/AMPK signaling. *Cell Death Dis.* 10, 615. doi: 10.1038/s41419-019-1844-2

Kim, E. J., Kwon, K. A., Lee, Y. E., Kim, J. H., Kim, S. H., and Kim, J. H. (2018). Red Ginseng extract reduces hypoxia-induced epithelial-mesenchymal transition by repressing NF-κB and ERK1/2 pathways in colon cancer. *J. GINSENG Res.* 42, 288–297. doi: 10.1016/j.jgr.2017.03.008

Lastraioli, E., Guasti, L., Crociani, O., Polvani, S., Hofmann, G., Witchel, H., et al. (2004). herg1 gene and HERG1 protein are overexpressed in colorectal cancers and regulate cell invasion of tumor cells. *Cancer Res.* 64 (2), 606–611. doi: 10.1158/0008-5472.can-03-2360

Lastraioli, E., Bencini, L., Bianchini, E., Romoli, M. R., Crociani, O., Giommoni, E., et al. (2012). hERG1 Channels and Glut-1 as Independent Prognostic Indicators of Worse Outcome in Stage I and II Colorectal Cancer: A Pilot Study. *Transl. Oncol.* 5, 105–112. doi: 10.1593/tlo.11250

Lastraioli, E., Perrone, G., Sette, A., Fiore, A., Crociani, O., Manoli, S., et al. (2015a). b. hERG1 channels drive tumour malignancy and may serve as prognostic factor in pancreatic ductal adenocarcinoma. *Br. J. Cancer* 112, 1076–1087. doi: 10.1038/bjc.2015.28

Lastraioli, E., Iorio, J., and Arcangeli, A. (2015b). a. Ion channel expression as promising cancer biomarker. *Biochim. Biophys. Acta* 1848, 2685–2702. doi: 10.1016/j.bbamem.2014.12.016

Lehenkari, P. P., and Horton, M. A. (1999). Single integrin molecule adhesion forces in intact cells measured by atomic force microscopy. *Biochem. Biophys. Res. Commun.* 259, 645–650. doi: 10.1006/bbrc.1999.0827

Ludwig, F. T., Schwab, A., and Stock, C. (2013). The Na+/H+ -exchanger (NHE1) generates pH nanodomains at focal adhesions. *J. Cell. Physiol.* 228, 1351–1358. doi: 10.1002/jcp.24293

Manoli, S., Coppola, S., Duranti, C., Lulli, M., Magni, L., Kuppalu, N., et al. (2019). The Activity of Kv 11.1 Potassium Channel Modulates F-Actin Organization During Cell Migration of Pancreatic Ductal Adenocarcinoma Cells. *Cancers (Basel).* 11, 135. doi: 10.3390/cancers11020135

Martìn-Padura, I., Bazzoni, G., Zanetti, A., Bernasconi, S., Elices, M. J., Mantovani, A., et al. (1994). A novel mechanism of colon carcinoma cell adhesion to the endothelium triggered by beta 1 integrin chain. *J. Biol. Chem.* 269, 6124–6132.

Masereel, B., Pochet, L., and Laeckmann, D. (2003). An overview of inhibitors of Na(+)/H(+) exchanger. *Eur. J. Med. Chem.* 38, 547–554. doi: 10.1016/s0223-5234(03)00100-4

Masi, A., Becchetti, A., Restano-Cassulini, R., Polvani, S., Hofmann, G., Buccoliero, A. M., et al. (2005). hERG1 channels are overexpressed in glioblastoma multiforme and modulate VEGF secretion in glioblastoma cell lines. *Br. J. Cancer* 93, 781–792. doi: 10.1038/sj.bjc.6602775

Mboge, M. Y., Mahon, B. P., McKenna, R., and Frost, S. C. (2018). Carbonic Anhydrases: Role in pH Control and Cancer. *Metabolites* 8, 19. doi: 10.3390/metabo8010019

McCarty, M. F., and Whitaker, J. (2010). Manipulating tumor acidification as a cancer treatment strategy. *Altern. Med. Rev.* 15, 264–272.

McIntyre, A., Patiar, S., Wigfield, S., Li, J. L., Ledaki, I., Turley, H., et al. (2012). Carbonic anhydrase IX promotes tumor growth and necrosis in vivo and inhibition enhances anti-VEGF therapy. *Clin. Cancer Res.* 18, 3100–3111. doi: 10.1158/1078-0432.CCR-11-1877

Orlowski, J., and Grinstein, S. (2004). Diversity of the mammalian sodium/proton exchanger SLC9 gene family. *Pflugers Arch.* 447, 549–565. doi: 10.1007/s00424-003-1110-3

Paradiso, A., Cardone, R. A., Bellizzi, A., Bagorda, A., Guerra, L., Tommasino, M., et al. (2004). The Na+–H+ exchanger-1 induces cytoskeletal changes involving reciprocal RhoA and Rac1 signaling, resulting in motility and invasion in MDA-MB-435 cells. *Breast Cancer Res.* 6, R616–R628. doi: 10.1186/bcr922

Parkkila, S., Rajaniemi, H., Parkkila, A. K., Kivela, J., Waheed, A., Pastorekova, S., et al. (2000). Carbonic anhydrase inhibitor suppresses invasion of renal cancer cells in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 97, 2220–2224. doi: 10.1073/pnas.040554897

Patel, H., and Barber, D. L. (2005). A developmentally regulated Na–H exchanger in Dictyostelium discoideum is necessary for cell polarity during chemotaxis. *J. Cell Biol.* 169, 321–329. doi: 10.1083/jcb.200412145

Pelillo, C., Bergamo, A., Mollica, H., Bestagno, M., and Sava, G. (2015). Colorectal Cancer Metastases Settle in the Hepatic Microenvironment Through α5β1 Integrin. *J. Cell Biochem.* 116, 2385–2396. doi: 10.1002/jcb.25189

Persi, E., Duran-Frigola, M., Damaghi, M., Roush, W. R., Aloy, P., Cleveland, J. L., LQ., et al. (2018). System analysis of intracellular pH vulnerabilities for cancer therapy. *Nat. Commun.* 9, 2997. doi: 10.1038/s41467-018-05261-x

Pillozzi, S., and Arcangeli, A. (2009). "Physical and Functional Interaction between Integrins and hERG1 Channels in Cancer Cells," in *Integrins and Ion Channels: Molecular Complexes and Signaling*. Ed. A. Becchetti and A Arcangeli (Berlin, Germany: Landes Bioscience and Springer Science+Business Media).

Pillozzi, S., D'Amico, M., Bartoli, G., Gasparoli, L., Petroni, G., Crociani, O., et al. (2018). The combined activation of $K_{Ca}3.1$ and inhibition of $K_v11.1$/hERG1 currents contribute to overcome Cisplatin resistance in colorectal cancer cells. *Br. J. Cancer* 118, 200–212. doi: 10.1038/bjc.2017.392

Pinheiro, C., Longatto-Filho, A., Azevedo-Silva, J., Casal, M., Schmitt, F. C., and Baltazar, F. (2012). Role of monocarboxylate transporters in human cancers: state of the art. *J. Bioenerg. Biomembr.* 44, 127. doi: 10.1007/s10863-012-9428-1

Plopper, G. E., McNamee, H. P., Dike, L. E., Bojanowski, K., and Ingber, D. E. (1995). Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. *Mol. Biol. Cell.* 6, 1349–1365. doi: 10.1091/mbc.6.10.1349

Putney, L. K., Denker, S. P., and Barber, D. L. (2002). The changing face of the Na +/H+ exchanger, NHE1: structure, regulation, and cellular actions. *Annu. Rev. Pharmacol. Toxicol.* 42, 527–552. doi: 10.1146/annurev.pharmtox.42.092001.143801

Schmidt, R., Streit, M., Kaiser, R., Herzberg, F., Schirner, M., Schramm, K., et al. (1998). *De novo* expression of the α5β1-fibronectin receptor in HT29 colon-cancer cells reduces activity of c-src. Increase of c-src activity by attachment on fibronectin. *Int. J. Cancer* 76, 91–98. doi: 10.1002/(sici)1097-0215(19980330)76:1<91::aid-ijc15>3.0.co;2-j

Schwartz, M. A., Both, G., and Lechene, C. (1989). Effect of cell spreading on cytoplasmic pH in normal and transformed fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* 86, 4525–4529. doi: 10.1073/pnas.86.12.4525

Schwartz, M. A., Ingber, D. E., Lawrence, M., Springer, T. A., and Lechene, C. (1991). Share the Ability to Induce Elevation of Intracellular pH. *Exp. Cell Res.* 195, 533–535. doi: 10.1016/0014-4827(91)90407-L

Sennoune, S. R., Luo, D., and Martinez-Zaguilán, R. (2004). Plasmalemmal vacuolar-type H^+-ATPase in cancer biology. *Cell Biochem. Biophys.* 40, 185. doi: 10.1385/CBB:40:2:18

Sharma, M., Astekar, M., Soi, S., Manjunatha, B. S., Shetty, D. C., and Radhakrishnan, R. (2015). pH Gradient Reversal: An Emerging Hallmark of Cancers. *Recent Patents Anti-Cancer Drug Discov.* 10, 244–258. doi: 10.2174/1574892810666150708110608

Srivastava, J., Barreiro, G., Groscurth, S., Gingras, A. R., Goult, B. T., Critchley, D. R., et al. (2008). Structural model and functional significance of pH-dependent talin–actin binding for focal adhesion remodeling J. *PNAS* 105, 14436–14441. doi: 10.1073/pnas.0805163105

Stock, C., and Pedersen, S. (2017). Roles of pH and the Na^+/H^+ exchanger NHE1 in cancer: From cell biology and animal models to an emerging translational perspective? *Semin. Cancer Biol.* 43, 5–16. doi: 10.1016/j.semcancer.2016.12.001

Stock, C., and Schwab, A. (2006). Role of the Na/H exchanger NHE1 in cell migration. *Acta Physiol. (Oxf)* 187, 149–157. doi: 10.1111/j.1748-1716.2006.01543.x

Stock, C., and Schwab, A. (2009). Protons make tumor cells move like clockwork. *Pflugers Arch.* 458, 981–992. doi: 10.1007/s00424-009-0677-8

Stock, C., Gassner, B., Hauck, C. R., Arnold, H., Mally, S., Eble, J. A., et al. (2005). Migration of human melanoma cells depends on extracellular pH and Na+/H+ exchange. *J. Physiol.* 567, 225–238. doi: 10.1113/jphysiol.2005.088344

Stock, C., Cardone, R. A., Busco, G., Krahling, H., Schwab, A., and Reshkin, S. J. (2008). Protons extruded by NHE1: digestive or glue? *Eur. J. Cell Biol.* 87, 591–599. doi: 10.1016/j.ejcb.2008.01.007

Webb, B. A., Chimenti, M., Jacobson, M. P., and Barber, D. L. (2011). Dysregulated pH: a perfect storm for cancer progression. *Nat. Rev. Cancer* 11, 671–677. doi: 10.1038/nrc3110

Yatohgo, T., Izumi, M., Kashiwagi, H., and Hayashi, M. (1988). Novel purification of vitronectin from human plasma by heparin affinity chromatography. *Cell Struct. Funct.* 13, 281–292. doi: 10.1247/csf.13.281

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 Iorio, Duranti, Lottini, Lastraioli, Bagni, Becchetti and Arcangeli. 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 Pharmacology

MINI REVIEW
published: 18 June 2020
doi: 10.3389/fphar.2020.00913

Voltage-Gated K^+/Na^+ Channels and Scorpion Venom Toxins in Cancer

Alexis Díaz-García[1,2*] *and Diego Varela*[2,3*]

[1] LifEscozul Chile SpA, Santiago, Chile, [2] Millennium Nucleus of Ion Channel-Associated Diseases (MiNICAD), Universidad de Chile, Santiago, Chile, [3] Program of Physiology and Biophysics, Faculty of Medicine, Institute of Biomedical Sciences (ICBM), Universidad de Chile, Santiago, Chile

Ion channels have recently been recognized as novel therapeutic targets in cancer research since they are overexpressed in different histological tissues, and their activity is linked to proliferation, tumor progression, angiogenesis, metastasis, and apoptosis. Voltage gated-potassium channels (VGKC) are involved in cell proliferation, cancer progression, cell cycle transition, and apoptosis. Moreover, voltage-dependent sodium channels (VGSC) contribute to decreases in extracellular pH, which, in turn, promotes cancer cell migration and invasion. Furthermore, VGSC and VGKC modulate voltage-sensitive Ca^{2+} channel activity by controlling the membrane potential and regulating Ca^{2+} influx, which functions as a second messenger in processes related to proliferation, invasion, migration, and metastasis. The subgroup of these types of channels that have shown a high oncogenic potential have become known as "oncochannels", and the evidence has highlighted them as key potential therapeutic targets. Scorpion venoms contain a high proportion of peptide toxins that act by modulating voltage-gated Na^+/K^+ channel activity. Increasing scientific data have pointed out that scorpion venoms and their toxins can affect the activity of oncochannels, thus showing their potential for anticancer therapy. In this review, we provide an update of the most relevant voltage-gated $Na^+\backslash K^+$ ion channels as cellular targets and discuss the possibility of using scorpion venom and toxins for anticancer therapy.

Keywords: cancer, ion channels, scorpion venom, toxins, voltage-dependent

OPEN ACCESS

Edited by:
Sébastien Roger, Université de Tours, France

Reviewed by:
Christian Legros, Université d'Angers, France
Stephan Kellenberger, University of Lausanne, Switzerland

***Correspondence:**
Alexis Díaz-García
alexisdg76@gmail.com
Diego Varela
dvarela@uchile.cl

Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *02 January 2020*
Accepted: *04 June 2020*
Published: *18 June 2020*

Citation:
Díaz-García A and Varela D (2020) Voltage-Gated K^+/Na^+ Channels and Scorpion Venom Toxins in Cancer. Front. Pharmacol. 11:913. doi: 10.3389/fphar.2020.00913

ION CHANNELS AND CANCER

Ion channels are critical regulators of cellular homeostasis in excitable and non-excitable cells, regulating vital physiological processes, such as electrical signal transmission, gene expression, cell signaling pathways, hormonal secretion, learning, and memory (Bates, 2015). During oncogenic transformation, cancer cells acquire aberrant characteristics with respect to their normal counterparts, which represent the core of cancer hallmarks, such as self-sustained proliferation, tumor progression, angiogenesis, metastasis, and apoptosis resistance (Bates, 2015; Prevarskaya et al., 2018). Many genes encoding ion channels are targets of oncogenic transformation, as previously reported (Prevarskaya et al., 2018). In turn, these gene products contribute to the development of one or more cancer hallmarks, promoting the transition to a more aggressive cancer phenotype; this is exemplified by the positive correlation between ion channel overexpression and

functional dysregulation with tumor progression, invasion, and metastasis (Litan and Langhans, 2015; Prevarskaya et al., 2018). The amount of evidence showcasing abnormal ion channel activity linked to carcinogenesis, cancer migration, and invasion has led to consideration of cancer as a channelopathy (Litan and Langhans, 2015; Prevarskaya et al., 2018).

In cancer, the expression changes of ion channels can be related to early diagnosis, prediction of disease aggressiveness, or as markers that allow monitoring of the response to treatment (Lastraioli et al., 2015; Kischel et al., 2019). Different ion channel subfamilies have been associated with a great variety of cancers from different histological origins and even with particular stages of cancer initiation and progression (Rao et al., 2015; Kischel et al., 2019).

In the present article, we focus on voltage-dependent K^+- and Na^+-channels as these are the main targets of scorpion venom in prey capture and self-defense behaviors (Quintero-Hernández et al., 2013).

K^+-CHANNELS IN CANCER

K^+-channels control K^+ permeability, and play crucial roles in both excitable and non-excitable cells (Kuang et al., 2015). Voltage-dependent K^+-channels constitute the largest and most diverse group of voltage-gated ion channels expressed in cells and comprise a pore-forming subunit ($K_V\alpha$ subunit) that may associate with auxiliary $K_V\beta$ subunits (Tian et al., 2014; Kuang et al., 2015). The $K_V\beta$ subunits modify ion channel function and/or localization and increase the diversity of physiological roles associated with these ion channels, with implications in health and disease (Tian et al., 2014; Serrano-Novillo et al., 2019). The scientific literature shows a considerable amount of information indicating the role of K^+-channels in cell proliferation, cancer progression (Wulff and Castle, 2010; Ouadid-Ahidouch et al., 2016), and migration (Chow et al., 2018), and at least four different mechanisms have been proposed (**Figure 1**), and discussed in-depth in recent dedicated reviews (Huang and Jan, 2014; Pardo and Stühmer, 2014).

In cancer cells, there are significant alterations in the expression of K^+-channels, which is manifested not only by the increase in their total expression, but also in the relative proportion of their different subtypes (Jiang et al., 2017; Zavala et al., 2019). The most prominent ion channel subfamilies present in primary tumors and metastases include Kv, Ether-à-go-go (EAG), and K_{Ca} (Tian et al., 2014; Kuang et al., 2015). Kv10.1, Kv11.1, $K_{Ca}1.1$, and Kv1.3 are the most investigated ion channels, due to their cancer hallmark-related properties. Their implication in preclinical and clinical behavior related to different cancer stages raises them as potential targets for therapy (**Table 1**) (Comes et al., 2015; Prevarskaya et al., 2018).

$K_V11.1$ (also known as the human Ether-à-go-go (hERG) channel) is probably the most studied ion channel in the EAG subfamily. In normal healthy tissues, its expression is usually low. In contrast, this ion channel is expressed in a higher proportion in leukemia, ovarian, lung, and breast cancer cells, among others (Jehle et al., 2011). $K_V11.1$ channels have notable participation in the cell cycle and appear as regulators of apoptosis and cell

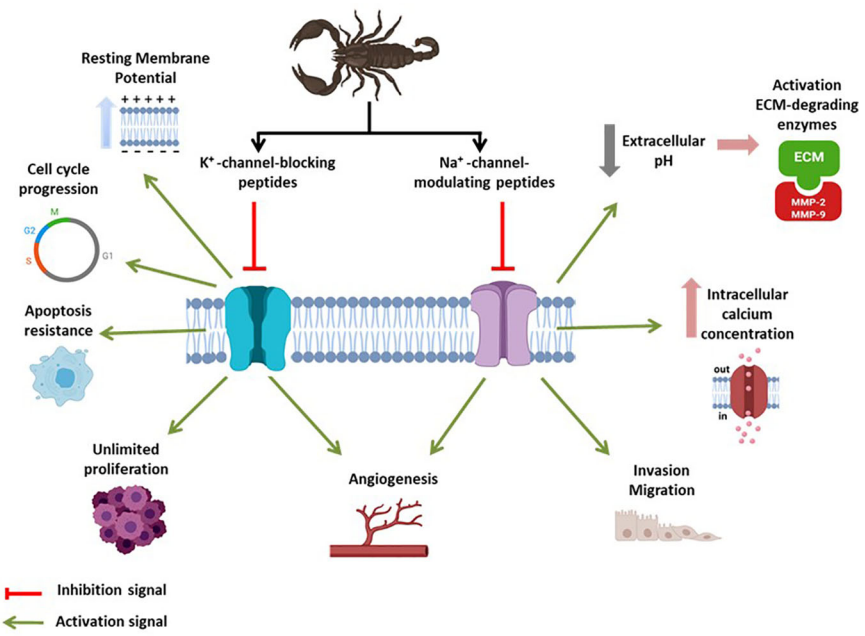


FIGURE 1 | The global effect of scorpion toxins on cancer-related voltage-gated K^+/Na^+-channels. An "activation signal" (green) indicates the pathological feature of ion channel activity in the context of cancer development. An "inhibition signal" (red) indicates inhibitory action of scorpion toxins, meaning cancer-hallmark inhibition.

TABLE 1 | Main characteristics of the most studied cancer-related K^+/Na^+-channels and their recognized modulating toxins.

Ion channel	Characteristics	Localization		Expression level		Biological Activity*		Scorpion toxin modulators	Ref
		Normal tissue	Cancer tissue	Normal tissue	Cancer tissue	Normal tissue	Cancer tissue		
K_V11.1	Voltage-dependent activation-inactivation/rapid delayed rectifier	Colon (smooth muscle), pancreas, uterus, kidney, blood, brain, heart	Leukemia, ovarian, lung, breast, colon, gastric, brain, skin, prostate	Low expression	Overexpression	Action potential repolarization (heart), firing frequency and hormone release (endocrine cells), excitability (CNS)	Cell cycle, cell proliferation, apoptosis, migration, invasion	CsEKerg1	(Jehle et al., 2011; Arcangeli and Becchetti, 2015; Cubeddu, 2016; Goversen et al., 2019)
K_V10.1	Voltage-gated non-inactivating delayed rectifier channel/Calmodulin inhibition	Hypothalamus, hippocampus, cerebral cortex, cerebellum, olfactory nerve	Cervix, lung, breast, colon, ovarian, neuroblastoma, liver, prostate, glioma, gastric, head and neck, squamous cell carcinoma	Low expression	Overexpression	Activation of? excitable cells, signal transduction, hormone secretion regulation, intracellular osmoregulation	Ion flux-independent mechanism for migration, cell cycle G1-G2/M progression, intracellular pathways, cell proliferation, tumor progression	κ-Hefutoxin 1	(Martínez et al., 2015; Ouadid-Ahidouch et al., 2016; Cázares-Ordoñez and Pardo, 2017; Wang et al., 2017)
K_V1.3	K_V1.3 (cell membrane) Voltage-dependent activation-inactivation/ delayed rectifier channel	Hypothalamus, olfactory bulb, immune cells, kidney, colon	Breast, colon, smooth muscle, skeletal muscle, lymph node, B cells	Low expression	Overexpression	Resting membrane potential setting, signal transduction, cell proliferation, volume regulation	Proliferation through driving force for Ca^{2+}, migration	KAaH1, KAaH2, charybdotoxin, margatoxin, maurotoxin	(Pérez-Verdaguer et al., 2016; Leanza et al., 2017; Venturini et al., 2017; Serrano-Albarrás et al., 2018)
	mK_V1.3 (inner mitochondrial membrane) Voltage dependent activation-inactivation				Overexpression/ Low expression in apoptotic resistant cancers	Mitochondrial membrane potential regulation	Apoptosis, ROS production, cell proliferation, intracellular signaling pathways		(Leanza et al., 2017; Checchetto et al., 2019)
K_V1.1	Voltage-dependent activation-inactivation	Central and Peripheral Nervous Systems (hippocampus, cerebellum, neocortex, peripheral nerves)	Glioblastoma, neuroblastoma, breast, colon adenocarcinoma, lung, cervix	Low expression	Overexpression	Control of firing frequency, regulate action potential repolarization, regulate neurotransmitter release	Tumor progression, mitochondrial metabolism, migration	KAaH1, KAaH2	(Leanza et al., 2014; Liu et al., 2019; D'Adamo et al., 2020)
K_{Ca}1.1	Voltage-dependent activation/Ca^{2+}-modulated	skeletal muscles, nervous system, epithelium endocrine/ exocrine glands, endothelial vascular cells, smooth muscle cells	Somatostatinoma, endometrial, prostate, pituitary, breast, glioblastoma, neuroblastoma	Low expression	Overexpression	Modulation of calcium-signaling processes	ERK1/2 signaling, proliferation, migration, metastasis, apoptosis	Iberiotoxin, charybdotoxin	(Oeggerli et al., 2012; Contreras et al., 2013; Du et al., 2016; Li et al., 2018)
nNaV1.5	Voltage-dependent activation–inactivation	Skeletal muscle, heart	Breast	Adult variant (NaV1.5)	Neonatal variant (nNaV1.5)	Action potential generation and propagation	Migration, invasion, metastasis	unknown	(Driffort et al., 2014; Nelson et al., 2015a; Nelson et al., 2015b; Yamaci et al., 2017;

(Continued)

TABLE 1 | Continued

Ion channel	Characteristics	Localization		Expression level		Biological Activity*		Scorpion toxin modulators	Ref
		Normal tissue	Cancer tissue	Normal tissue	Cancer tissue	Normal tissue	Cancer tissue		
									Djamgoz et al., 2019)
NaV1.6	Voltage-dependent activation–inactivation	CNS neurons	Cervix, colorectal, astrocytoma	Low expression	Overexpression	Action potential generation and propagation	Migration, invasion, metastasis	Cn2, AaHIV	(Lopez-Charcas et al., 2018; Guan et al., 2018)
NaV1.7	Voltage-dependent activation–inactivation	PNS neurons, adrenal gland, endocrine pancreatic cells	Prostate, lung, gastrointestinal tract	Low expression	Overexpression	Action potential generation and propagation	Migration, invasion, metastasis	unknown	(Campbell et al., 2013; Shan et al., 2014; Xia et al., 2016; Chen et al., 2019)

**Referred to both positive and negative correlation respect to ion channel-regulated physiological and biological activity.*

proliferation in cancer cells (Staudacher et al., 2014; Arcangeli and Becchetti, 2015). In the heart, Kv11.1 is key for cardiac repolarization and therefore, its off-target inhibition induces long QT syndrome. Thus, safety pharmacological studies include K_V11.1 channel assays as the primary test, decreasing its practical impact as an anticancer therapy-related target (Goversen et al., 2019).

K_V10.1 channel is selectively expressed in brain areas (**Table 1**). However, this channel is overexpressed in more than 70% of tumors and in cancer cell lines from the cervix, lung, breast, ovary, neuroblast, liver, prostate, glial cells, and gastrointestinal tract (Martínez et al., 2015; Wang et al., 2017). Moreover, its crucial role in tumorigenesis, cell signaling, cell cycle, and tumor growth has been recognized (Ouadid-Ahidouch et al., 2016). Different experimental approaches have demonstrated the relationship between K_V10.1 channel blockage and anticancer effects, including induction of apoptosis, inhibition of cell proliferation, and delay in tumor growth (Cázares-Ordoñez and Pardo, 2017), suggesting that this channel is a promising candidate as a tumor and therapeutic marker in oncology.

K_{Ca}1.1 channel is ubiquitously expressed in human tissues such as skeletal muscle and the nervous system, with the exception of cardiac myocytes. K_{Ca}1.1 channels regulate calcium influx into cells and thereby modulate Ca^{2+}-signaling processes (Contreras et al., 2013). This channel is overexpressed in cancer cell lines from prostate, glia, breast, pancreas, and endometrium (**Table 1**) (Du et al., 2014; Du et al., 2016; Klumpp et al., 2016; Li et al., 2018; Noda et al., 2020). In the prostate, K_{Ca}1.1 channel overexpression regulates proliferation and migration (Du et al., 2016) and in breast cancer, its overexpression has been associated with advanced tumor stage, high tumor cell proliferation, and poor prognosis (Oeggerli et al., 2012).

K_V1.3 channel is mostly expressed in neurons and immune cells (Pérez-Verdaguer et al., 2016). It is located at the plasma membrane, sets the resting membrane potential (RMP) and regulates cell proliferation and cell volume. Furthermore, this channel is also located in the inner mitochondrial membrane (mK_V1.3), where it plays a role in apoptotic signaling (Teisseyre et al., 2019) (**Table 1**). Overexpression of K_V1.3 channels is observed in breast, colon, smooth muscle, skeletal muscle, and lymph node cancers (Teisseyre et al., 2015; Teisseyre et al., 2019). Its plasma membrane expression is associated with controlling cell proliferation by inducing a transitory hyperpolarization necessary to augment the driving force for Ca^{2+} influx during G1/S progression (Serrano-Albarrás et al., 2018). Moreover, mK_V1.3 channels play a role in drug-induced apoptosis by mechanisms that sensitize cancer cells (Pérez-Verdaguer et al., 2016). The potential role of K_V1.3 channels as cancer therapy targets has been recently evidenced in *in vitro* and *in vivo* experimental models of glioblastoma, melanoma, and pancreatic adenocarcinoma, where mK_V1.3 inhibition induces apoptotic cell death *in vitro* (Leanza et al., 2017; Venturini et al., 2017; Checchetto et al., 2019). All these pieces of evidence promoted K_V1.3 channels as attractive potential molecular targets in both cancer diagnostics and therapy (Comes et al., 2015; Prevarskaya et al., 2018).

Notwithstanding that the ion channels mentioned above represent some of the most prominent ones in cancer; other voltage-gated ion channels linked to cancer proliferation and progression are upregulated in some tumors and have been described in dedicated reviews (Huang and Jan, 2014; Serrano-Novillo et al., 2019).

NA^+-CHANNELS IN CANCER

Voltage-dependent sodium channels (VGSC) are transmembrane proteins that are generally expressed in excitable cells, although they are also found, to a limited extent, in non-excitable cells (Catterall, 2012; Erickson et al., 2018). There are nine pore-forming α-subunits of sodium channels, $Na_V1.1$-$Na_V1.9$, encoded by the genes SCN1A-SCN11A. The pore-forming α-subunit comprises four highly similar transmembrane domains (I-IV), each composed of six transmembrane segments (S1–S6). The first four transmembrane segments of each domain constitute the voltage sensor domain, and the last two form the pore domain (Catterall, 2012). The α-subunit properties can be modulated in a subtype-specific manner, by association with one or more than one smaller auxiliary β-subunit ($Na_V\beta_{1-4}$); conferring tissue-specific expression patterns, varying voltage dependent activation and inactivation, and increasing functional channel density at the plasma membrane (Catterall, 2017).

The oncogenic transformation of VGSC can contribute to the development of one or more cancer hallmarks, promoting the transition to more aggressive cancer phenotypes, as previously reported (Prevarskaya et al., 2018); this is particularly exemplified by the positive correlation between VGSC overexpression and functional dysregulation with invasion/migration and metastatic potential (Andrikopoulos et al., 2011; Djamgoz et al., 2019; Mao et al., 2019) (**Table 1**).

Proliferating and cancer cells show a RMP between -10 to -50 mV, compared to normal and non-proliferating cells (-50 to -90 mV) (Yang and Brackenbury, 2013). This RMP range fits with the window current range for VGSC, meaning that although the majority of VGSCs will be inactivated, the small percentage of non-inactivated channels will lead to a persistent Na^+-current, increasing the $[Na]_i$ (Yang and Brackenbury, 2013). The augmented intracellular Na^+ concentration leads to an increased intracellular Ca^{2+} concentration, either by promoting the reverse mode of the Na^+/Ca^{2+} exchanger (NCX) or by inducing plasma membrane depolarization and consequent activation of voltage-sensitive Ca^{2+} channels (VGCC) (Patel and Brackenbury, 2015; Roger et al., 2015). Both mechanisms, driven directly or indirectly by VGSC, might be considered relevant for cancer migration and invasion. However, there are very few reports providing experimental evidence about the functional link between VGSC, NCX, and VGCC (Besson et al., 2015; Angus and Ruben, 2019; Rodrigues et al., 2019) and this aspect needs broader investigation.

A hallmark of a tumor´s extracellular space is a more acidic environment than in normal healthy tissues (pH 6.2–6.8 instead of pH 7.2–7.4), as a consequence of the predominant glycolytic metabolism of cancer cells; this particular extracellular environment enhances the degradation of the extracellular matrix by favoring Cathepsin B and S activation, and thus, promotes cell migration (Besson et al., 2015; Angus and Ruben, 2019). This extracellular acidification is dependent on Na^+/H^+ exchanger 1 (NHE1), which in turn depends on the $[Na^+]$ transmembrane gradient (Besson et al., 2015; Angus and Ruben, 2019). Given the increased $[Na]_i$, a reduced NHE1 activity should be expected; however, two hypotheses have been suggested to explain this apparent contradiction. i) that these channels allosterically regulate NHE1 by inducing a higher rate of H^+ extrusion at neutral pHi ranges, and ii) that the expression of VGSC in late endosome vesicles is responsible for the extra-acidification of these vesicles (Besson et al., 2015; Angus and Ruben, 2019). In this last scenario, the extracellular acidic environment would be a consequence of vesicle release.

Tetrodotoxin (TTX) is a toxin, mainly associated with fishes of the *Tetraodontidae* family, that specifically blocks a subgroup of VGSCs and inhibits the migration and invasion of cancer cells, indicating that cell motility requires Na^+-channel activity (Nelson et al., 2015a) a feature mainly associated with overexpression of the neonatal variants of $Na_V1.5$ ($nNa_V1.5$), $Na_V1.6$, and $Na_V1.7$ (Roger et al., 2015; Mao et al., 2019).

$nNa_V1.5$ overexpression was initially identified in the metastatic human breast cancer cell line MDA-MB-231 and breast biopsy samples (**Table 1**) (Yamaci et al., 2017). Later, the same positive correlation was found between the expression of $nNa_V1.5$ channels and the high invasive potential of cancer cells from diverse histological origins (Djamgoz et al., 2019), suggesting that the overexpression of $nNa_V1.5$ channel is necessary and sufficient to increase the metastatic potential of cancer cells (Nelson et al., 2015b).

$Na_V1.6$ is overexpressed in cervical cancer biopsies, cancer cell lines, and primary cultures positive for the human papillomavirus (**Table 1**). In these cases, a $Na_V1.6$ splice variant with preferential cytoplasmatic localization is expressed (Lopez-Charcas et al., 2018). Overexpression of $Na_V1.6$ protein is associated with invasive status in cervical cancer and low-grade astrocytoma, mediated through increased MMP-2 activity (Lopez-Charcas et al., 2018; Guan et al., 2018).

$Na_V1.7$ is ectopically expressed in particular types of cancers (**Table 1**) (Campbell et al., 2013; Xia et al., 2016; Chen et al., 2019). In gastric cancer, this channel is associated with poor patient outcomes by promoting cell invasion through the modulation of H^+ efflux (Xia et al., 2016). In rat prostate cancer, $Na_V1.7$ channel activity promotes the activation of p38/NF-κβ, and Rho GTPase signaling pathways as a linking node for controlling cellular motility, cell adhesion, and vesicular trafficking (Chen et al., 2019). In non-small cell lung cancer, the $Na_V1.7$ channel is overexpressed in metastatic cells by more than 60% when compared to their non-metastatic counterparts (Campbell et al., 2013).

Independent of their function as auxiliary subunits, $Na_V\beta_{1-3}$ are overexpressed in different cancers and have been associated with increased cellular motility, invasion, and metastasis (O'Malley and Isom, 2015; Bouza and Isom, 2018). Additionally, $Na_V\beta_1$ has been linked to tumor growth, increase

of vascular endothelial growth factor secretion, and angiogenesis (O'Malley and Isom, 2015; Bouza and Isom, 2018). In contrast, $Na_V\beta_3$ functions as a tumor suppressor by inducing p53-dependent apoptosis when overexpressed (Bouza and Isom, 2018). Thus, the $Na_V\beta$-subunits are interesting and poorly explored potential targets for cancer therapy, needing an in-depth investigation to identify their complete clinical and physiopathological relevance.

Overall, VGSCs and $Na_V\beta$ are up-regulated in numerous types of metastatic cancer cells and play important roles in regulating cell migration and invasion in solid tumors. Therefore, they can be considered as key regulators of cancer development and the metastatic cascade (Mao et al., 2019). The noncanonical activity of VGSC that regulates other cancer hallmarks (*i.e.,* cell proliferation) is scarcely understood and needs to be investigated with more detail (Black and Waxman, 2013).

SCORPION VENOM AND THEIR TOXINS IN CANCER

Worldwide, there are more than 2,200 scorpion species, grouped in 19 families (Ward et al., 2018). The scorpion venom is a complex mixture containing a great variety of proteins with molecular weights between 3 kDa and 90 kDa, which constitute most of the components. The main biological activity of the scorpion venom is due to the presence of low molecular weight peptide toxins of basic nature, which are highly cross-linked (3–4 disulfide bridges) (Quintero-Hernández et al., 2013; Kuzmenkov et al., 2015). These peptides exhibit different pharmacological and toxicological activities (Quintero-Hernández et al., 2013; Kuzmenkov et al., 2015). Until now, only a few scorpion species have been experimentally tested as anticancer agents, mainly for cancer cells from solid tumors and to a lesser extent, for hematopoietic cancers (Raposo, 2017).

In only two cases (*B. martensii* and *R. junceus*), the scientific results correlate with the experiences in traditional medicine and with the low toxicity recognized in toxicological experiments in mice (Wang and Ji, 2005; Diaz-Garcia et al., 2019a; Díaz-García et al., 2019b). The anticancer effect of *B. martensii* scorpion venom has been tested successfully against human glioma U251-MG by using rodent xenograft models (Wang and Ji, 2005). Likewise, *in vivo* toxicological studies have been carried out, using *R. junceus* venom administered through intraperitoneal (10 mg/kg) or oral (2,000 mg/kg) routes, and toxic effects have not been observed (Garcia-Gomez et al., 2011; Lagarto et al., 2020). Pharmacokinetic and biodistribution studies carried out on breast tumor-bearing mice administered with a single dose (12.5 mg/kg), by intravenous or oral routes, showed that medium residence time (MRT) of venom in tumor tissue was higher than in the remaining organs tested, suggesting a high selectivity for tumor tissue, adding to their antitumor effect (Diaz-Garcia et al., 2019a). Additionally, breast tumor-bearing mice injected intraperitoneally with ten consecutive doses of *R. junceus* venom (3.2 mg/kg), showed reduced tumor progression and reduction of Ki67 and CD31 tumor markers, confirming its anticancer potential (Díaz-García et al., 2019b). Two additional scorpion species, *Androctonus amoreuxi* (Salem et al., 2016) and *Leiurus quinquestriatus* (Al Asmari and Khan, 2016), have been tested with some favorable *in vivo* anticancer effects, even though both are two of the most dangerous species (Ward et al., 2018). These overall promising results have focused the scientific research on the isolation and identification of the components responsible for the anticancer effects of scorpion venoms.

Peptides recognizing K^+- and Na^+-channels are prominent in scorpion venoms, constituting more than 75% of all peptide/proteins (de Oliveira et al., 2018; Cid-Uribe et al., 2019). Most peptides recognizing K^+ channels are pore-blocking peptides and some of them have been studied in the context of cancer (**Table 1**). For example, KAaH1, a $K_V1.1$ and $K_V1.3$ blocker, and KAaH2, a $K_V1.1$ blocker, both derived from the *Androctonus australis Hector* venom, have shown anticancer potential (Aissaoui et al., 2018). KAaH1 inhibits migration and adhesion of different cancer cells, whereas KAaH2 inhibits the proliferation of gliomas (Aissaoui et al., 2018). Evidence indicates that iberiotoxin inhibits cell proliferation, migration, and invasion in breast and endometrial cancer cell lines, due to its blocking effects on BK channels (Schickling et al., 2015; Li et al., 2018); while charybdotoxin, a known blocker of $K_{Ca}3.1$, $K_V1.3$, and BK channels, inhibits proliferation and cell cycle progression in pancreatic and endometrial cancer cell lines (Jager et al., 2004; Schickling et al., 2015; Li et al., 2018). Both toxins were isolated from the *Leiurus quinquestriatus* scorpion. Similarly, margatoxin (MgTX), a peptide isolated from *Centruroides margaritatus*, is a selective $K_V1.3$-blocker that reduces cell proliferation, and tumor progression, decreases the expression of cell cycle regulators and increases the expression level of proapoptotic proteins in cancer experimental models (Jang et al., 2011). CsEKerg1 toxin, from the *Centruroides sculpturatus* scorpion has been evaluated as a hERG current inhibitor in an *in vitro* cancer model, suggesting its potential use in Kv11.1 channel-overexpressing cancer cells (Nastainczyk et al., 2002); this result opens a window of opportunity for other Kv11.1-blocking toxins described until now (Jimenez-Vargas et al., 2012). κ-Hefutoxin 1 from *Heterometrus fulvipes* scorpion venom (Moreels et al., 2017) has been identified as the first toxin recognizing $K_V10.1$ channels, without affecting other voltage-gated K^+-channels (Moreels et al., 2017). Moreover, maurotoxin isolated from *Scorpio maurus palmatus* scorpion can block various potassium channels, including SK, IK, $K_V1.1$, and $K_V1.3$, some of which have been recognized as cancer-related ion channels (Castle et al., 2003). Tapamin, a toxin isolated from the *Mesobuthus tamulus* scorpion, can block some cancer-related ion channels, such as SK and $K_{Ca}3.1$, and exerts a cytotoxic effect on cancer cells (Pedarzani et al., 2002; Ramirez-Cordero et al., 2014).

Although Na^+-channel-modulating peptides represent the highest percentage among all scorpion venom-derived toxins (Cid-Uribe et al., 2019), the identification of scorpion venom peptides that interact with metastatic-related Na^+ channels has been difficult, and only three cases have been identified (**Table 1**).

Cn2, a β-toxin from *Centruroides noxius Hoffmann* scorpion venom, modulates $Na_V1.6$ activity in F11 neuroblastoma cells (Escalona et al., 2014). In cell culture, Cn2 reduces proliferation by increasing cells at the SubG1 and G0/G1 stages, leading to apoptosis induction (Escalona et al., 2014). This toxin binds to the receptor site 4, located in the S3–S4 and S1–S2 extracellular loops of the VGSC channel domain II, enhancing channel activation by shifting the voltage-dependence of channel activation to the left, as a consequence of voltage-sensor trapping (Cestele et al., 1998), and reducing the Na^+ current peak amplitude (Pedraza Escalona and Possani, 2013). AGAP, isolated from *Buthus martensii*, is an α-toxin that interacts with Na^+-channels. Evidence suggests that AGAP affects the translation of the $Na_V\beta1$ subunit in cancer cells and has been successfully evaluated against Ehrlich ascites tumor and S-180 fibrosarcoma models *in vivo*. Furthermore, this peptide can inhibit cancer cell stemness, epithelial-mesenchymal transition (EMT), migration, and invasion in MCF-7 and MDA-MB-231 human breast cancer cells *in vitro* and tumor growth *in vivo* (Guo et al., 2016; Kampo et al., 2019). Finally, AaHIV toxin, isolated from *Androctonus australis* venom, is a Na^+ channel-modulating toxin active against cancer cells (BenAissa et al., 2019). AaHIV can interact with the extracellular loops of segments S1–S2 in the voltage sensor domain, prolonging the inactivation recovery time of Nav1.6 channels, and inhibiting cancer cell proliferation in a dose-dependent manner (BenAissa et al., 2019). Unlike anti-migratory and anti-metastatic properties, the antiproliferative properties of Na^+-channel-interacting scorpion toxins represent an unexpected feature that should be deeply investigated. There is no doubt that scorpion venom peptide toxins inhibit the functional activity of voltage-gated K^+/Na^+-channels, reducing their impact on the hallmark of cancer (**Figure 1**).

It is worth mentioning that Chlorotoxin is the only toxin from scorpion venom that has been successfully evaluated in cancer preclinical and clinical trials (Cohen-Inbar and Zaaroor, 2016; Mahadevappa et al., 2017; Cohen et al., 2018). However, this toxin recognizes voltage-dependent Cl^- channels (Dardevet et al., 2015), which was not within the scope of this review.

CONCLUDING REMARKS

Evidence indicates that upregulation of voltage-dependent K^+ and Na^+ channels is linked to cancer hallmarks. Thus, they have become key player as new alternatives to be used as diagnostic, prognostic, and therapeutic targets in cancer. Scorpion venoms contain small peptides acting either at the cell membrane or intracellularly, and even cross the blood-brain barrier. The mechanisms of action of scorpion venom toxins described here, related to ion channel-modulating effects, give new insights to the plethora of potential new mechanisms of action that could be discovered from scorpion venom peptides. Laboratories dedicated to scorpion venom research have usually described the anticancer effects of scorpion venom and/or components for the first time; far away from the anticancer drug development programs and their resources. There is no doubt that the inclusion of these natural products, such as plant extracts, as part of the anticancer drug discovery programs, might increase the arsenal of active components as potential new drugs against relatively new targets. Importantly, the interaction of both research areas might represent a substantial qualitative leap that could open a highway of promising alternatives to be used as adjuvant therapeutic approaches or conventional treatment in anticancer therapy.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to the writing and preparation of the manuscript.

FUNDING

The Millennium Nucleus of Ion Channel-Associated Diseases (MiNICAD) is a Millennium Nucleus supported by the Iniciativa Científica Milenio of the Ministry of Economy, Development, and Tourism (Chile). This work was supported by Vicerrectoría de Investigación y Desarrollo, Universidad de Chile (VID-Enlace, ENL24/19).

REFERENCES

Aissaoui, D., Mlayah-Bellalouna, S., Jebali, J., Abdelkafi-Koubaa, Z., Souid, S., Moslah, W., et al. (2018). Functional role of Kv1.1 and Kv1.3 channels in the neoplastic progression steps of three cancer cell lines, elucidated by scorpion peptides. *Int. J. Biol. Macromol.* 111, 1146–1155. doi: 10.1016/j.ijbiomac.2018.01.144

Al Asmari, A. K., and Khan, A. Q. (2016). Investigation of in vivo potential of scorpion venom against skin tumorigenesis in mice via targeting markers associated with cancer development. *Drug Des. Devel. Ther.* 10, 3387–3397. doi: 10.2147/DDDT.S113171

Andrikopoulos, P., Fraser, S. P., Patterson, L., Ahmad, Z., Burcu, H., Ottaviani, D., et al. (2011). Angiogenic functions of voltage-gated Na+ Channels in human endothelial cells: modulation of vascular endothelial growth factor (VEGF) signaling. *J. Biol. Chem.* 286 (19), 16846–16860. doi: 10.1074/jbc.M110.187559

Angus, M., and Ruben, P. (2019). Voltage gated sodium channels in cancer and their potential mechanisms of action. *Channels (Austin)* 13 (1), 400–409. doi: 10.1080/19336950.2019.1666455

Arcangeli, A., and Becchetti, A. (2015). Novel perspectives in cancer therapy: Targeting ion channels. *Drug Resist. Update* 21-22, 11–19. doi: 10.1016/j.drup.2015.06.002

Bates, E. (2015). Ion channels in development and cancer. *Annu. Rev. Cell Dev. Biol.* 31, 231–247. doi: 10.1146/annurev-cellbio-100814-125338

BenAissa, R., Othman, H., Villard, C., Peigneur, S., Mlayah-Bellalouna, S., Abdelkafi-Koubaa, Z., et al. (2019). AaHIV a sodium channel scorpion toxin inhibits the proliferation of DU145 prostate cancer cells. *Biochem. Biophys. Res. Commun.* 521 (2), 340–346. doi: 10.1016/j.bbrc.2019.10.115

Besson, P., Driffort, V., Bon, E., Gradek, F., Chevalier, S., and Roger, S. (2015). How do voltage-gated sodium channels enhance migration and invasiveness in cancer cells? *Biochim. Biophys. Acta* 1848 (10 Pt B), 2493–2501. doi: 10.1016/j.bbamem.2015.04.013

Black, J. A., and Waxman, S. G. (2013). Noncanonical roles of voltage-gated sodium channels. *Neuron* 80 (2), 280–291. doi: 10.1016/j.neuron.2013.09.012

Bouza, A. A., and Isom, L. L. (2018). Voltage-Gated Sodium Channel beta Subunits and Their Related Diseases. *Handb. Exp. Pharmacol.* 246, 423–450. doi: 10.1007/164_2017_48

Cázares-Ordoñez, V., and Pardo, L. (2017). Kv10.1 potassium channel: from the brain to the tumors. *Biochem. Cell Biol.* 95 (5), 531–536. doi: 10.1139/bcb-2017-0062

Campbell, T., Main, M., and Fitzgerald, E. (2013). Functional expression of the voltage-gated Na^+-channel Nav1.7 is necessary for EGF-mediated invasion in human non-small cell lung cancer cells. *J. Cell Sci.* 126 ((Pt 21), 4939–4949. doi: 10.1242/jcs.130013

Castle, N. A., London, D. O., Creech, C., Fajloun, Z., Stocker, J. W., and Sabatier, J. M. (2003). Maurotoxin: a potent inhibitor of intermediate conductance Ca2+-activated potassium channels. *Mol. Pharmacol.* 63 (2), 409–418. doi: 10.1124/mol.63.2.409

Catterall, W. (2012). Voltage-gated sodium channels at 60: structure, function and pathophysiology. *J. Physiol.* 590 (11), 2577–2589. doi: 10.1113/jphysiol.2011.224204

Catterall, W. (2017). Forty Years of Sodium Channels: Structure, Function, Pharmacology, and Epilepsy. *Neurochem. Res.* 42 (9), 2495–2504. doi: 10.1007/s11064-017-2314-9

Cestele, S., Qu, Y., Rogers, J. C., Rochat, H., Scheuer, T., and Catterall, W. A. (1998). Voltage sensor-trapping: enhanced activation of sodium channels by beta-scorpion toxin bound to the S3-S4 loop in domain II. *Neuron* 21 (4), 919–931. doi: 10.1016/s0896-6273(00)80606-6

Checchetto, V., Prosdocimi, E., and Leanza, L. (2019). Mitochondrial Kv1.3: a New Target in Cancer Biology? *Cell Physiol. Biochem.* 53 (S1), 52–62. doi: 10.33594/000000195

Chen, B., Zhang, C., Wang, Z., Chen, Y., Xie, H., Li, S., et al. (2019). Mechanistic insights into Nav1.7-dependent regulation of rat prostate cancer cell invasiveness revealed by toxin probes and proteomic analysis. *FEBS J.* 286 (13), 2549–2561. doi: 10.1111/febs.14823

Chow, L., Cheng, K., Wong, K., and Leung, Y. (2018). Voltage-gated K+ channels promote BT-474 breast cancer cell migration. *Chin. J. Cancer Res.* 30 (6), 613–622. doi: 10.21147/j.issn.1000-9604.2018.06.06

Cid-Uribe, J. I., Meneses, E. P., Batista, C. V. F., Ortiz, E., and Possani, L. D. (2019). Dissecting Toxicity: The Venom Gland Transcriptome and the Venom Proteome of the Highly Venomous Scorpion Centruroides limpidus (Karsch, 1879). *Toxins (Basel)* 11 (5), 247. doi: 10.3390/toxins11050247

Cohen, G., Burks, S. R., and Frank, J. A. (2018). Chlorotoxin-A Multimodal Imaging Platform for Targeting Glioma Tumors. *Toxins (Basel)* 10 (12), 496. doi: 10.3390/toxins10120496

Cohen-Inbar, O., and Zaaroor, M. (2016). Glioblastoma multiforme targeted therapy: The Chlorotoxin story. *J. Clin. Neurosci.* 33, 52–58. doi: 10.1016/j.jocn.2016.04.012

Comes, N., Serrano-Albarrás, A., Capera, J., Serrano-Novillo, C., Condom, E., Ramón, Y., et al. (2015). Involvement of potassium channels in the progression of cancer to a more malignant phenotype. *Biochim. Biophys. Acta* 1848 (10), 2477–2492. doi: 10.1016/j.bbamem.2014.12.008

Contreras, G. F., Castillo, K., Enrique, N., Carrasquel-Ursulaez, W., Castillo, J. P., Milesi, V., et al. (2013). A BK (Slo1) channel journey from molecule to physiology. *Channels (Austin)* 7 (6), 442–458. doi: 10.4161/chan.26242

Cubeddu, L. (2016). Drug-induced Inhibition and Trafficking Disruption of ion Channels: Pathogenesis of QT Abnormalities and Drug-induced Fatal Arrhythmias. *Curr. Cardiol. Rev.* 12 (2), 141–154. doi: 10.2174/1573403X12666160301120217

D'Adamo, M. C., Liantonio, A., Rolland, J. F., Pessia, M., and Imbrici, P. (2020). Kv1.1 Channelopathies: Pathophysiological Mechanisms and Therapeutic Approaches. *Int. J. Mol. Sci.* 21 (8), 2935. doi: 10.3390/ijms21082935

Dardevet, L., Rani, D., Aziz, T. A., Bazin, I., Sabatier, J. M., Fadl, M., et al. (2015). Chlorotoxin: a helpful natural scorpion peptide to diagnose glioma and fight tumor invasion. *Toxins (Basel)* 7 (4), 1079–1101. doi: 10.3390/toxins7041079

de Oliveira, U. C., Nishiyama, M. Y.Jr., Dos Santos, M. B. V., Santos-da-Silva, A. P., Chalkidis, H. M., Souza-Imberg, A., et al. (2018). Proteomic endorsed transcriptomic profiles of venom glands from Tityus obscurus and T. serrulatus scorpions. *PloS One* 13 (3), e0193739. doi: 10.1371/journal.pone.0193739

Diaz-Garcia, A., Pimentel Gonzalez, G., Basaco Bernabeu, T., Rodriguez Aurrecochea, J. C., Rodriguez Sanchez, H., Sanchez Monzon, I., et al. (2019a). Pharmacokinetics and Biodistribution of Rhopalurus junceus Scorpion Venom in Tumor-Bearing Mice after Intravenous and Oral Administration. *Iran BioMed. J.* 23 (4), 287–296. doi: 10.29252/ibj.23.4.287

Díaz-García, A., Ruiz-Fuentes, J. L., Frión-Herrera, Y., Yglesias-Rivera, A., Riquenez Garlobo, Y., Rodríguez Sánchez, H., et al. (2019b). Rhopalurus junceus scorpion venom induces antitumor effect in vitro and in vivo against a murine mammary adenocarcinoma model. *Iranian J. Basic Med. Sci.* 22 (7), 759–765. doi: 10.22038/ijbms.2019.33308.7956

Djamgoz, M., Fraser, S., and Brackenbury, W. (2019). In Vivo Evidence for Voltage-Gated Sodium Channel Expression in Carcinomas and Potentiation of Metastasis. *Cancers (Basel)* 11 (11), E1675. doi: 10.3390/cancers11111675

Driffort, V., Gillet, L., Bon, E., Marionneau-Lambot, S., Oullier, T., Joulin, V., et al. (2014). Ranolazine inhibits NaV1.5-mediated breast cancer cell invasiveness and lung colonization. *Mol. Cancer* 13, 264. doi: 10.1186/1476-4598-13-264

Du, C., Chen, L., Zhang, H., Wang, Z., Liu, W., Xie, X., et al. (2014). Caveolin-1 limits the contribution of BKCa channel to MCF-7 breast cancer cell proliferation and invasion. *Int. J. Mol. Sci.* 15 (11), 20706–20722. doi: 10.3390/ijms151120706

Du, C., Zheng, Z., Li, D., Chen, L., Li, N., Yi, X., et al. (2016). BKCa promotes growth and metastasis of prostate cancer through facilitating the coupling between alphavbeta3 integrin and FAK. *Oncotarget* 7 (26), 40174–40188. doi: 10.18632/oncotarget.9559

Erickson, A., Deiteren, A., Harrington, A., Garcia-Caraballo, S., Castro, J., Caldwell, A., et al. (2018). Voltage-gated sodium channels: (NaV)igating the field to determine their contribution to visceral nociception. *J. Physiol.* 596 (5), 785–807. doi: 10.1113/JP273461

Escalona, M. P., Batista, C. V., Cassulini, R. R., Rios, M. S., Coronas, F. I., and Possani, L. D. (2014). A proteomic analysis of the early secondary molecular effects caused by Cn2 scorpion toxin on neuroblastoma cells. *J. Proteom.* 111, 212–223. doi: 10.1016/j.jprot.2014.04.035

Garcia-Gomez, B. I., Coronas, F. I., Restano-Cassulini, R., Rodriguez, R. R., and Possani, L. D. (2011). Biochemical and molecular characterization of the venom from the Cuban scorpion Rhopalurus junceus. *Toxicon* 58 (1), 18–27. doi: 10.1016/j.toxicon.2011.04.011

Goversen, B., Jonsson, M., van den Heuvel, N., Rijken, R., Vos, M., van Veen, T., et al. (2019). The influence of hERG1a and hERG1b isoforms on drug safety screening in iPSC-CMs. *Prog. Biophys. Mol. Biol.* S0079-6107 (18), 30263–30263. doi: 10.1016/j.pbiomolbio.2019.02.003

Guan, G., Zhao, M., Xu, X., Boczek, T., Mao, X., Li, Z., et al. (2018). Abnormal changes in voltage-gated sodium channels subtypes NaV1.1, NaV1.2, NaV1.3, NaV1.6 and CaM/CaMKII pathway in low-grade astrocytoma. *Neurosci. Lett.* 674, 148–155. doi: 10.1016/j.neulet.2018.03.047

Guo, G., Cui, Y., Chen, H., Zhang, L., Zhao, M., Chen, B., et al. (2016). Analgesic-antitumor peptide inhibits the migration and invasion of HepG2 cells by an upregulated VGSC beta1 subunit. *Tumour Biol.* 37 (3), 3033–3041. doi: 10.1007/s13277-015-4067-x

Huang, X., and Jan, L. (2014). Targeting potassium channels in cancer. *J. Cell Biol.* 206 (2), 151–162. doi: 10.1083/jcb.201404136

Jager, H., Dreker, T., Buck, A., Giehl, K., Gress, T., and Grissmer, S. (2004). Blockage of intermediate-conductance Ca2+-activated K+ channels inhibit human pancreatic cancer cell growth in vitro. *Mol. Pharmacol.* 65 (3), 630–638. doi: 10.1124/mol.65.3.630

Jang, S. H., Choi, S. Y., Ryu, P. D., and Lee, S. Y. (2011). Anti-proliferative effect of Kv1.3 blockers in A549 human lung adenocarcinoma in vitro and in vivo. *Eur. J. Pharmacol.* 651 (1-3), 26–32. doi: 10.1016/j.ejphar.2010.10.066

Jehle, J., Schweizer, P., Katus, H., and Thomas, D. (2011). Novel roles for hERG K(+) channels in cell proliferation and apoptosis. *Cell Death Dis.* 18 (2), e193. doi: 10.1038/cddis.2011.77

Jiang, S., Zhu, L., Yang, J., Hu, L., Gu, J., Xing, X., et al. (2017). Integrated expression profiling of potassium channels identifys KCNN4 as a prognostic biomarker of pancreatic cancer. *Biochem. Biophys. Res. Commun.* 494 (1-2), 113–119. doi: 10.1016/j.bbrc.2017.10.072

Jimenez-Vargas, J. M., Restano-Cassulini, R., and Possani, L. D. (2012). Toxin modulators and blockers of hERG K(+) channels. *Toxicon* 60 (4), 492–501. doi: 10.1016/j.toxicon.2012.03.024

Kampo, S., Ahmmed, B., Zhou, T., Owusu, L., Anabah, T. W., Doudou, N. R., et al. (2019). Scorpion Venom Analgesic Peptide, BmK AGAP Inhibits Stemness, and Epithelial-Mesenchymal Transition by Down-Regulating PTX3 in Breast Cancer. *Front. Oncol.* 9, 21. doi: 10.3389/fonc.2019.00021

Kischel, P., Girault, A., Rodat-Despoix, L., Chamlali, M., Radoslavova, S., Abou Daya, H., et al. (2019). Ion Channels: New Actors Playing in Chemotherapeutic Resistance. *Cancers (Basel)* 11 (3), E376. doi: 10.3390/cancers11030376

Klumpp, L., Sezgin, E. C., Eckert, F., and Huber, S. M. (2016). Ion Channels in Brain Metastasis. *Int. J. Mol. Sci.* 17 (9), 1513. doi: 10.3390/ijms17091513

Kuang, Q., Purhonen, P., and Hebert, H. (2015). Structure of potassium channels. *Cell Mol. Life Sci.* 72 (19), 3677–3693. doi: 10.1007/s00018-015-1948-5

Kuzmenkov, A., Grishin, E., and Vassilevski, A. (2015). Diversity of Potassium Channel Ligands: Focus on Scorpion Toxins. *Biochem. (Mosc)* 80 (13), 1764–1799. doi: 10.1134/S0006297915130118

Lagarto, A., Bueno, V., Perez, M. R., Rodriguez, C. C., Guevara, I., Valdes, O., et al. (2020). Safety evaluation of the venom from scorpion Rhopalurus junceus: Assessment of oral short term, subchronic toxicity and teratogenic effect. *Toxicon* 176, 59–66. doi: 10.1016/j.toxicon.2020.02.002

Lastraioli, E., Iorio, J., and Arcangeli, A. (2015). Ion channel expression as promising cancer biomarker. *Biochim. Biophys. Acta* 1848 (10), 2685–2702. doi: 10.1016/j.bbamem.2014.12.016

Leanza, L., O'Reilly, P., Doyle, A., Venturini, E., Zoratti, M., Szegezdi, E., et al. (2014). Correlation between potassium channel expression and sensitivity to drug-induced cell death in tumor cell lines. *Curr. Pharm. Des.* 20 (2), 189–200. doi: 10.2174/13816128113199990032

Leanza, L., Romio, M., Becker, K., Azzolini, M., Trentin, L., Managò, A., et al. (2017). Direct Pharmacological Targeting of a Mitochondrial Ion Channel Selectively Kills Tumor Cells In Vivo. *Cancer Cell* 31 (4), 516–531. doi: 10.1016/j.ccell.2017.03.003

Li, N., Liu, L., Li, G., Xia, M., Du, C., and Zheng, Z. (2018). The role of BKCa in endometrial cancer HEC-1-B cell proliferation and migration. *Gene* 655, 42–47. doi: 10.1016/j.gene.2018.02.055

Litan, A., and Langhans, S. A. (2015). Cancer as a channelopathy: ion channels and pumps in tumor development and progression. *Front. Cell Neurosci.* 9, 86. doi: 10.3389/fncel.2015.00086

Liu, L., Chen, Y., Zhang, Q., and Li, C. (2019). Silencing of KCNA1 suppresses the cervical cancer development via mitochondria damage. *Channels (Austin)* 13 (1), 321–330. doi: 10.1080/19336950.2019.1648627

Lopez-Charcas, O., Espinosa, A., Alfaro, A., Herrera-Carrillo, Z., Ramirez-Cordero, B., Cortes-Reynosa, P., et al. (2018). The invasiveness of human cervical cancer associated to the function of NaV1.6 channels is mediated by MMP-2 activity. *Sci. Rep.* 8 (1), 12995. doi: 10.1038/s41598-018-31364-y

Mahadevappa, R., Ma, R., and Kwok, H. F. (2017). Venom Peptides: Improving Specificity in Cancer Therapy. *Trends Cancer* 3 (9), 611–614. doi: 10.1016/j.trecan.2017.07.004

Mao, W., Zhang, J., Körner, H., Jiang, Y., and Ying, S. (2019). The Emerging Role of Voltage-Gated Sodium Channels in Tumor Biology. *Front. Oncol.* 9, 124. doi: 10.3389/fonc.2019.00124

Martínez, R., Stühmer, W., Martin, S., Schell, J., Reichmann, A., Rohde, V., et al. (2015). Analysis of the expression of Kv10.1 potassium channel in patients with brain metastases and glioblastoma multiforme: impact on survival. *BMC Cancer* 15, 839. doi: 10.1186/s12885-015-1848-y

Moreels, L., Peigneur, S., Yamaguchi, Y., Vriens, K., Waelkens, E., Zhu, S., et al. (2017). Expanding the pharmacological profile of kappa-hefutoxin 1 and analogues: A focus on the inhibitory effect on the oncogenic channel Kv10.1. *Peptides* 98, 43–50. doi: 10.1016/j.peptides.2016.08.008

Nastainczyk, W., Meves, H., and Watt, D. D. (2002). A short-chain peptide toxin isolated from Centruroides sculpturatus scorpion venom inhibits ether-a-go-go-related gene K(+) channels. *Toxicon* 40 (7), 1053–1058. doi: 10.1016/S0041-0101(02)00100-9

Nelson, M., Yang, M., Millican-Slater, R., and Brackenbury, W. (2015a). Nav1.5 regulates breast tumor growth and metastatic dissemination in vivo. *Oncotarget* 6 (32), 32914–32929. doi: 10.18632/oncotarget.5441

Nelson, M., Yang, M., Dowle, A., Thomas, J., and Brackenbury, W. (2015b). The sodium channel-blocking antiepileptic drug phenytoin inhibits breast tumour growth and metastasis. *Mol. Cancer* 14, 13. doi: 10.1186/s12943-014-0277-x

Noda, S., Chikazawa, K., Suzuki, Y., Imaizumi, Y., and Yamamura, H. (2020). Involvement of the gamma1 subunit of the large-conductance Ca(2+)-activated K(+) channel in the proliferation of human somatostatinoma cells. *Biochem. Biophys. Res. Commun.* 525 (4), 1032–1037. doi: 10.1016/j.bbrc.2020.02.176

O'Malley, H. A., and Isom, L. L. (2015). Sodium channel beta subunits: emerging targets in channelopathies. *Annu. Rev. Physiol.* 77, 481–504. doi: 10.1146/annurev-physiol-021014-071846

Oeggerli, M., Tian, Y., Ruiz, C., Wijker, B., Sauter, G., Obermann, E., et al. (2012). Role of KCNMA1 in breast cancer. *PloS One* 7 (8), e41664. doi: 10.1371/journal.pone.0041664

Ouadid-Ahidouch, H., Ahidouch, A., and Pardo, L. (2016). Kv10.1 K(+) channel: from physiology to cancer. *Pflugers Arch.* 468 (5), 751–762. doi: 10.1007/s00424-015-1784-3

Pérez-Verdaguer, M., Capera, J., Serrano-Novillo, C., Estadella, I., Sastre, D., and Felipe, A. (2016). The voltage-gated potassium channel Kv1.3 is a promising multitherapeutic target against human pathologies. *Expert Opin. Ther. Targets.* 20 (5), 577–591. doi: 10.1517/14728222.2016.1112792

Pardo, L., and Stühmer, W. (2014). The roles of K(+) channels in cancer. *Nat. Rev. Cancer* 14 (1), 39–48. doi: 10.1038/nrc3635

Patel, F., and Brackenbury, W. J. (2015). Dual roles of voltage-gated sodium channels in development and cancer. *Int. J. Dev. Biol.* 59 (7-9), 357–366. doi: 10.1387/ijdb.150171wb

Pedarzani, P., D'Hoedt, D., Doorty, K. B., Wadsworth, J. D., Joseph, J. S., Jeyaseelan, K., et al. (2002). Tamapin, a venom peptide from the Indian red scorpion (Mesobuthus tamulus) that targets small conductance Ca2+-activated K+ channels and afterhyperpolarization currents in central neurons. *J. Biol. Chem.* 277 (48), 46101–46109. doi: 10.1074/jbc.M206465200

Pedraza Escalona, M., and Possani, L. D. (2013). Scorpion beta-toxins and voltage-gated sodium channels: interactions and effects. *Front. Biosci. (Landmark Ed.)* 18, 572–587. doi: 10.2741/4121

Prevarskaya, N., Skryma, R., and Shuba, Y. (2018). Ion Channels in Cancer: Are Cancer Hallmarks Oncochannelopathies? *Physiol. Rev.* 98 (2), 559–621. doi: 10.1152/physrev.00044.2016

Quintero-Hernández, V., Jiménez-Vargas, J., Gurrola, G., Valdivia, H., and Possani, L. (2013). Scorpion venom components that affect ion-channels function. *Toxicon* 76, 328–342. doi: 10.1016/j.toxicon.2013.07.012

Ramirez-Cordero, B., Toledano, Y., Cano-Sanchez, P., Hernandez-Lopez, R., Flores-Solis, D., Saucedo-Yanez, A. L., et al. (2014). Cytotoxicity of recombinant tamapin and related toxin-like peptides on model cell lines. *Chem. Res. Toxicol.* 27 (6), 960–967. doi: 10.1021/tx4004193

Rao, V., Perez-Neut, M., Kaja, S., and Gentile, S. (2015). Voltage-gated ion channels in cancer cell proliferation. *Cancers (Basel)* 7 (2), 849–875. doi: 10.3390/cancers7020813

Raposo, C. (2017). Scorpion and spider venoms in cancer treatment: state of the art, challenges, and perspectives. *J. Clin. Transl. Res.* 3 (2), 233–249. doi: 10.18053/jctres.03.201702.002

Rodrigues, T., Estevez, G. N. N., and Tersariol, I. (2019). Na(+)/Ca(2+) exchangers: Unexploited opportunities for cancer therapy? *Biochem. Pharmacol.* 163, 357–361. doi: 10.1016/j.bcp.2019.02.032

Roger, S., Gillet, L., Le Guennec, J. Y., and Besson, P. (2015). Voltage-gated sodium channels and cancer: is excitability their primary role? *Front. Pharmacol.* 6, 152. doi: 10.3389/fphar.2015.00152

Salem, M. L., Shoukry, N. M., Teleb, W. K., Abdel-Daim, M. M., and Abdel-Rahman, M. A. (2016). In vitro and in vivo antitumor effects of the Egyptian scorpion Androctonus amoreuxi venom in an Ehrlich ascites tumor model. *Springerplus* 5, 570. doi: 10.1186/s40064-016-2269-3

Schickling, B. M., England, S. K., Aykin-Burns, N., Norian, L. A., Leslie, K. K., and Frieden-Korovkina, V. P. (2015). BKCa channel inhibitor modulates the tumorigenic ability of hormone-independent breast cancer cells via the Wnt pathway. *Oncol. Rep.* 33 (2), 533–538. doi: 10.3892/or.2014.3617

Serrano-Albarrás, A., Estadella, I., Cirera-Rocosa, S., Navarro-Pérez, M., and Felipe, A. (2018). Kv1.3: a multifunctional channel with many pathological implications. *Expert Opin. Ther. Targets.* 22 (2), 101–105. doi: 10.1080/14728222.2017.1420170

Serrano-Novillo, C., Capera, J., Colomer-Molera, M., Condom, E., Ferreres, J., and Felipe, A. (2019). Implication of Voltage-Gated Potassium Channels in Neoplastic Cell Proliferation. *Cancers (Basel)* 11 (3), E287. doi: 10.3390/cancers11030287

Shan, B., Dong, M., Tang, H., Wang, N., Zhang, J., Yan, C., et al. (2014). Voltage-gated sodium channels were differentially expressed in human normal prostate, benign prostatic hyperplasia and prostate cancer cells. *Oncol. Lett.* 8 (1), 345–350. doi: 10.3892/ol.2014.2110

Staudacher, I., Jehle, J., Staudacher, K., Pledl, H., Lemke, D., Schweizer, P., et al. (2014). HERG K+ channel-dependent apoptosis and cell cycle arrest in human glioblastoma cells. *PloS One* 9 (2), e88164. doi: 10.1371/journal.pone.0088164

Teisseyre, A., Gasiorowska, J., and Michalak, K. (2015). Voltage-Gated Potassium Channels Kv1.3–Potentially New Molecular Target in Cancer Diagnostics and Therapy. *Adv. Clin. Exp. Med.* 24 (3), 517–524. doi: 10.17219/acem/22339

Teisseyre, A., Palko-Labuz, A., Sroda-Pomianek, K., and Michalak, K. (2019). Voltage-Gated Potassium Channel Kv1.3 as a Target in Therapy of Cancer. *Front. Oncol.* 9, 933. doi: 10.3389/fonc.2019.00933

Tian, C., Zhu, R., Zhu, L., Qiu, T., Cao, Z., and Kang, T. (2014). Potassium channels: structures, diseases, and modulators. *Chem. Biol. Drug Des.* 83 (1), 1–26. doi: 10.1111/cbdd.12237

Venturini, E., Leanza, L., Azzolini, M., Kadow, S., Mattarei, A., Weller, M., et al. (2017). Targeting the Potassium Channel Kv1.3 Kills Glioblastoma Cells. *Neurosignals* 25 (1), 26–38. doi: 10.1159/000480643

Wang, W. X., and Ji, Y. H. (2005). Scorpion venom induces glioma cell apoptosis in vivo and inhibits glioma tumor growth in vitro. *J. Neurooncol.* 73 (1), 1–7. doi: 10.1007/s11060-004-4205-6

Wang, X., Chen, Y., Zhang, Y., Guo, S., Mo, L., An, H., et al. (2017). Eag1 Voltage-Dependent Potassium Channels: Structure, Electrophysiological Characteristics, and Function in Cancer. *J. Membr. Biol.* 250 (2), 123–132. doi: 10.1007/s00232-016-9944-8

Ward, M., Ellsworth, S., and Nystrom, G. (2018). A global accounting of medically significant scorpions: Epidemiology, major toxins, and comparative resources in harmless counterparts. *Toxicon* 151, 137–155. doi: 10.1016/j.toxicon.2018.07.007

Wulff, H., and Castle, N. (2010). Therapeutic potential of KCa3.1 blockers: recent advances and promising trends. *Expert Rev. Clin. Pharmacol.* 3 (3), 385–396. doi: 10.1586/ecp.10.11

Xia, J., Huang, N., Huang, H., Sun, L., Dong, S., Su, J., et al. (2016). Voltage-gated sodium channel Nav 1.7 promotes gastric cancer progression through MACC1-mediated upregulation of NHE1. *Int. J. Cancer* 139 (11), 2553–2369. doi: 10.1002/ijc.30381

Yamaci, R., Fraser, S., Battaloglu, E., Kaya, H., Erguler, K., Foster, C., et al. (2017). Neonatal Nav1.5 protein expression in normal adult human tissues and breast cancer. *Pathol. Res. Pract.* 213 (8), 900–907. doi: 10.1016/j.prp.2017.06.003

Yang, M., and Brackenbury, W. (2013). Membrane potential and cancer progression. *Front. Physiol.* 4, 185. doi: 10.3389/fphys.2013.00185

Zavala, W., Foscolo, M., Kunda, P., and Cavicchia, J. (2019). Changes in the expression of the potassium channels TASK1, TASK3 and TRESK in a rat model of oral squamous cell carcinoma and their relation to malignancy. *Arch. Biol.* 100, 75–85. doi: 10.1016/j.archoralbio.2019.02.007

Conflict of Interest: AD-G works for LifEscozul Chile SpA.

The remaining author declares 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 Díaz-García and Varela. 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 Pharmacology

REVIEW
published: 07 July 2020
doi: 10.3389/fphar.2020.00968

Calcium Permeable Channels in Cancer Hallmarks

Sendoa Tajada and Carlos Villalobos*

Instituto de Biología y Genética Molecular (IBGM), Universidad de Valladolid and Consejo Superior de Investigaciones Científicas (CSIC), Valladolid, Spain

Cancer, the second cause of death worldwide, is characterized by several common criteria, known as the "cancer hallmarks" such as unrestrained cell proliferation, cell death resistance, angiogenesis, invasion and metastasis. Calcium permeable channels are proteins present in external and internal biological membranes, diffusing Ca^{2+} ions down their electrochemical gradient. Numerous physiological functions are mediated by calcium channels, ranging from intracellular calcium homeostasis to sensory transduction. Consequently, calcium channels play important roles in human physiology and it is not a surprise the increasing number of evidences connecting calcium channels disorders with tumor cells growth, survival and migration. Multiple studies suggest that calcium signals are augmented in various cancer cell types, contributing to cancer hallmarks. This review focuses in the role of calcium permeable channels signaling in cancer with special attention to the mechanisms behind the remodeling of the calcium signals. Transient Receptor Potential (TRP) channels and Store Operated Channels (SOC) are the main extracellular Ca^{2+} source in the plasma membrane of non-excitable cells, while inositol trisphosphate receptors (IP_3R) are the main channels releasing Ca^{2+} from the endoplasmic reticulum (ER). Alterations in the function and/or expression of these calcium channels, as wells as, the calcium buffering by mitochondria affect intracellular calcium homeostasis and signaling, contributing to the transformation of normal cells into their tumor counterparts. Several compounds reported to counteract several cancer hallmarks also modulate the activity and/or the expression of these channels including non-steroidal anti-inflammatory drugs (NSAIDs) like sulindac and aspirin, and inhibitors of polyamine biosynthesis, like difluoromethylornithine (DFMO). The possible role of the calcium permeable channels targeted by these compounds in cancer and their action mechanism will be discussed also in the review.

Keywords: Ca^{2+} channels, cancer hallmarks, store-operated Ca^{2+} entry, TRP channels, calcium channel modulators in cancer

OPEN ACCESS

Edited by:
Sébastien Roger, Université de Tours, France

Reviewed by:
Juan Carlos Gomora, National Autonomous University of Mexico, Mexico
Dimitra Gkika, Lille University of Science and Technology, France

****Correspondence:***
Sendoa Tajada
sendoa.tajada@uva.es

Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *10 March 2020*
Accepted: *15 June 2020*
Published: *07 July 2020*

Citation:
Tajada S and Villalobos C (2020) Calcium Permeable Channels in Cancer Hallmarks. Front. Pharmacol. 11:968. doi: 10.3389/fphar.2020.00968

INTRODUCTION

Cancer is the second leading cause of death globally, causing 9.6 million deaths in 2018 with an increasing estimation of 70% over the next twenty years. There are more than 100 cancer types, affecting any part of the body in both sexes. However, the most common cancer types in women are: breast (25.4%), colorectal (9.6%), lung (8.8%), cervix (6.9%) and thyroid (6.3%), meanwhile lung

(15.5%), prostate (14.5%), colorectal (11.4%), liver (6.8%) and bladder (4.8%) are the most common among men (https://www.wcrf.org); (https://www.who.int). Every type of cancer is characterized by its unique clinical features, molecular markers and a disease-specific profile of genes. Nevertheless, all cancer types meet several common criteria, known as the "cancer hallmarks" described in 2000 by Hanahan and Weinberg (Hanahan and Weinberg, 2000). Although, new emerging cancer hallmarks were added in the last decade, the most accepted hallmarks are: (1) sustained proliferation, (2) cell death resistance, (3) tissue invasion and metastasis and (4) persistent angiogenesis.

Since the first hypothesis by the mid-1800s, that "canalis" are present in biological membranes (Brücke, 1847), numerous physiological functions have emerged mediated by ion channels, ranging from control cellular ionic homeostasis to sensory transduction. Consequently, ion channels perform key roles in human physiology and it is not a surprise the increasing number of evidences connecting channels disorders with a broader human diseases. Nowadays, abundant evidence demonstrate that calcium, calcium permeable channels and calcium signals, play important functions in cancer cells proliferation, apoptosis resistance, invasion and drug resistance, common cancer hallmarks. The first direct evidences connecting calcium channels and cancer were published in the 1980s, when independent studies confirmed the inhibition of cancer growth using particular calcium channels blockers (Lee et al., 1988; Batra and Alenfall, 1991; Taylor and Simpson, 1992). The ion channels research not only confirm the calcium channels correlation with cancer. Other ion channels with important roles in membrane potential or cell volume regulation, including Na^+, K^+ and Cl^- channels have been involved in the development of one or more cancer hallmarks (Pardo, 2004; Wang, 2004; Kunzelmann, 2005; Wulff et al., 2009; Schwab et al., 2012; Prevarskaya et al., 2018). At the present day, the relationship between altered expression and/or function of particular ion channels and cancer is well stablished, so well that the term "oncochannels" has been recently introduced (Huber, 2013).

This review focus in the role of calcium permeable channels signaling in cancer: from molecular mechanisms to therapeutics. Even though cancer therapies and drugs improved significantly in the last years, new and more efficient treatments are still needed to fight against such indiscriminate disease. Calcium channels and transporters are widely expressed in the plasma membrane of carcinoma cells where they may represent a good therapeutic target. For this reason, the study of ion channels role in both initial and advanced stages of the disease, should be useful to explore the possible clinical value of this membrane proteins as novel targets for therapy.

In this review we will consider the role of calcium permeable channels signaling in cancer with special attention to the mechanisms behind the remodeling of the calcium signals. The calcium channel modulators and their therapeutic use in treating cancer disease will be also discussed in the review.

CALCIUM PERMEABLE CHANNELS IN PLASMA MEMBRANE, ENDOPLASMIC RETICULUM AND MITOCHONDRIA

Calcium permeable channels are membrane proteins located in the external and internal cell membranes, including plasma membrane (PM), and endoplasmic reticulum (ER) or mitochondria, respectively. These channels diffuse passively Ca^{2+} ions down its electromechanical gradient from extracellular space and from intracellular calcium stores to the cytoplasm. At the PM there is a wide diversity of calcium channel types, characterized by their activation mechanism: (1) Voltage-gated calcium channels (VGCCs), (2) receptor-operated calcium channels (ROCCs), (3) store-operated calcium channels (SOCCs), (4) transient receptor potential channels (TRPs), (5) acid-sensing ion channels (ASICs) and (6) stretch-activated ion channels (SAICs). VGCCs are the major participants in the Ca^{2+} entry mechanism in excitable cells, including neurons, different types of muscle cells and some endocrine cells. In contrast, the main pathway for the Ca^{2+} influx in non-excitable cells such as epithelial cells from most carcinomas is performed by SOCCs (**Figure 1**). These channels generate the store-operated calcium entry (SOCE), originally called capacitative calcium entry. SOCCs open when ER Ca^{2+} stores are decreased from resting levels around 700 μM to about 200 μM (Alvarez and Montero, 2002; Villalobos et al., 2017). This partial depletion is sensed by the ER membrane-located Stromal Interaction Molecule 1 (STIM1) (Liou et al., 2005), when Ca^{2+} ions dissociate from its low affinity EF-Ca^{2+} binding domain. The decrease in ER-Ca^{2+} levels promotes STIM1 oligomerization and the interaction with the PM-localized calcium channel ORAI1 and/or TRP channels (Feske et al., 2006; Fahrner et al., 2017; Hernández-Morales et al., 2017; Secondo et al., 2018). The STIM1–ORAI1 interaction opens calcium release activated channels (CRAC), thus facilitating the calcium influx and the ER Ca^{2+}-stores refilling for subsequent stimuli (Villalobos et al., 2017). In mammals, ORAI1 channel has two other isoforms, ORAI2 and ORAI3 encoded by homologous genes (Feske et al., 2006) and STIM1 only one STIM2 (Putney, 2018). In addition, there are two variants of ORAI1, the longer ORAI1α and the shorter ORAI1β, due to different translation points, with different membrane diffusion coefficients (Fukushima et al., 2012). The three ORAI isoforms can be activated by STIM1 with some variations in the pharmacology and biophysical properties of the channel (Lis et al., 2007; DeHaven et al., 2008).

Ca^{2+} release mechanisms in the ER are mediated by two major Ca^{2+} permeable channels: (1) ryanodine receptors (RyRs) (Fleischer et al., 1985) and (2) inositol trisphosphate (IP_3) receptors (IP_3Rs) (Berridge, 1983; Streb et al., 1983) (**Figure 1**). RyRs open mainly when intracellular Ca^{2+} rises, thus acting as a ligand in a mechanism called, Ca^{2+}-induced Ca^{2+} release (CICR) (Endo et al., 1970). ER-Ca^{2+} release by IP_3Rs starts at the PM, where agonist-induced G-protein-coupled receptors (GPCRs) activates the phospholipase C (PLC) enzyme. The PLC selectively induces the hydrolysis of the

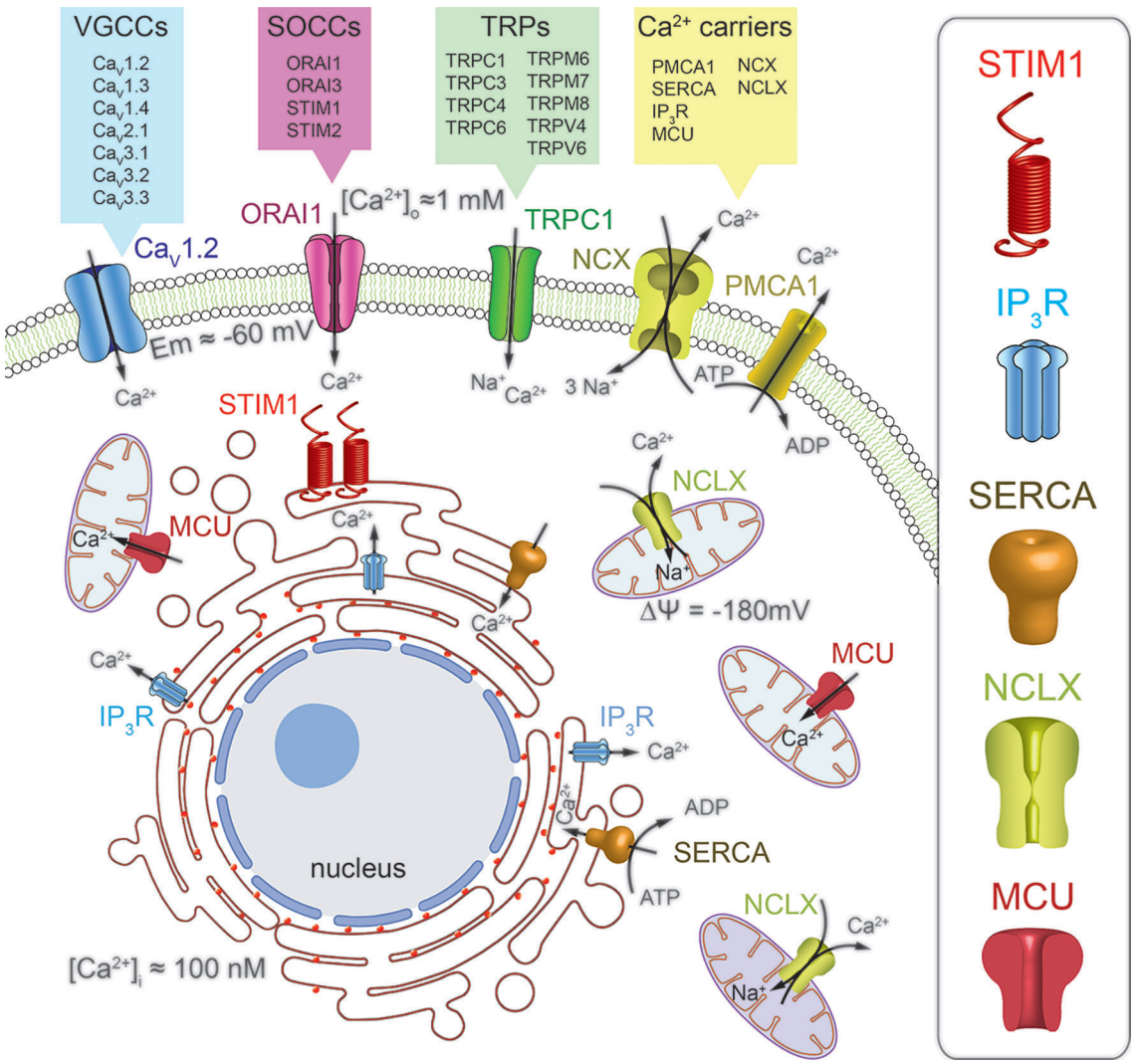


FIGURE 1 | Schematic representation of the main calcium signaling pathways through Ca^{2+} channels and transporters involved in the regulation of calcium homeostasis in mammalian cells. The calcium influx pathway from the extracellular space is mediated by the combined action of the voltage gated calcium channels (VGCCs), the store-operated calcium channels (SOCCs) and associated proteins (STIM) and the transient receptor potential (TRPs) Ca^{2+} permeable channels. The calcium release pathway from the endoplasmic reticulum (ER) and mitochondria is mediated by the inositol triphosphate receptors (IP_3R) and the mitochondrial Na^+/Ca^{2+} exchanger (NCLX), respectively. Furthermore, the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) and the mitochondrial Ca^{2+} uniporter (MCU) are the responsible for the free cytoplasmic Ca^{2+} sequestration into organelles. Finally, the Ca^{2+} extrusion pathway from the cytoplasm is regulated by the plasma membrane Ca^{2+}ATPase (PMCA1) and the Na^+/Ca^{2+} exchanger (NCX).

phosphatidylinositol 4,5-bisphosphate (PIP2), releasing diacylglycerol (DAG) and the second messenger IP_3, which diffuses to the ER activating its receptor (Berridge, 1987).

In mitochondria, the principal calcium channel is the mitochondrial calcium uniporter (MCU), a Ca^{2+}-activated, calcium channel, whose molecular machinery has been recently reported (Perocchi et al., 2010; Baughman et al., 2011; Sancak et al., 2013). MCU opens when large $[Ca^{2+}]$ microdomains develop at the mouth of this Ca^{2+}-operated, calcium channel. The huge mitochondrial potential ($\Delta\Psi$), close to −180 mV, promotes the Ca^{2+} removal from cytosol into mitochondria (Valero et al., 2008; Villalobos et al., 2017), clearing large cytosolic Ca^{2+} loads formed during VGCCs opening in excitable cells (Villalobos et al., 2001) or during Ca^{2+} release from the ER (Rizzuto et al., 1998). In various cellular types, SOCE regulation by mitochondria depends on the Ca^{2+} buffering capability of these organelles, being able to prevent the slow, Ca^{2+}-dependent inactivation of Ca^{2+} release activated calcium channels (CRAC) (Gilabert, 2000; Hernández-Morales et al., 2017).

CALCIUM PERMEABLE CHANNELS IN CANCER

Voltage-Gated Calcium Channels

Recent data provide evidence of the importance of the calcium permeable channels in cancer. Regarding VGCCs, altered gene expression profiles has been related with different cancer types (Wang et al., 2015a) and it has been proved that different calcium channels blockers prevent cancer invasion (Buchanan and McCloskey, 2016; Jacquemet et al., 2016).

Experimental and bioinformatic analysis showed that different subunits of VGCCs such as $Ca_V1.2$, $Ca_V1.3$, $Ca_V2.2$, $Ca_V3.1$, Cav3.2 and $Ca_V3.3$ are involved in the development and progression of different types of cancer, showing a significantly overexpression in breast cancer. $Ca_V1.4$ only showed up-regulation in testis cancer, whereas $Ca_V2.1$, $Ca_V1.2$ and $Ca_V1.3$ were deeply overexpressed in a wide range of cancer types (Wang et al., 2015a). More specifically, the high voltage-activated $Ca_V1.2$ channel, commonly localized in cardiac, skeletal, smooth muscle, neurons, fibroblasts and pancreatic cells (Lipscombe et al., 2004; Tajada et al., 2013; Dixon et al., 2015), is overexpressed in most cancer types, including colorectal, gastric, leukemia, brain, uterus, breast, pancreatic, sarcoma, skin and prostate (Wang et al., 2015a). The $Ca_V1.3$ is expressed in most of the same cells that express $Ca_V1.2$, as well as, neuroendocrine, amacrine cells, auditory hair cells, photoreceptors and the sinoatrial node where it contributes to pacemaking (Platzer et al., 2000; Lipscombe et al., 2004; Moreno et al., 2016). The $Ca_V1.3$ is highly expressed in most types of cancer, including breast and prostate cancer, but also in brain, colorectal, gastric, bladder, lung, esophageal and uterine tumors (Chen et al., 2014; Wang et al., 2015a; Buchanan and McCloskey, 2016; Jacquemet et al., 2016). See **Table 1** for a summary of calcium permeable channels involved in different cancer conditions. Furthermore, the carboxy terminus cleavage of both Cav1.2 and Cav1.3 promotes the altered expression of different proteins such as TRPV4 and small conductance potassium channels (SK3 and SK2) through the CCAT transcription factor or by direct translocation to the nucleus (Gomez-Ospina et al., 2006; Gomez-Ospina et al., 2013; Lu et al., 2015). Hence, additionally to the Ca^{2+} influx cancer-related signaling, both c-terminus can contribute to cancer hallmarks by modulation of other channels. It is also important to highlight the number of protein interaction sites described in the VGCCs, where different proteins modulate transcription factors such as CREB, NFAT and calmodulin, consequently linking ion channels with transcription factors involved in cancer progression (Mancini and Toker, 2009; Xiao et al., 2010; Berchtold and Villalobo, 2014).

Moreover, compelling studies point to the emerging role of VGCCs accessory subunits in cancer (Haworth and Brackenbury, 2019). The VGCCs are associated with these non-pore forming subunits that tune the biophysical properties of the channel, including activation, gating kinetics or trafficking to the PM (Dolphin, 2016). The increased expression of the $Ca_V1.x$ and the $Ca_V2.x$ accessory subunits, $\alpha_2\delta$ and β, have been related with different cancer hallmarks in liver, ovary, prostate, pancreas, lung, and colon tumors (Mitra et al., 2011; Cromer et al., 2015; Warnier et al., 2015; Yu et al., 2016; Badr et al., 2018; Gao et al., 2018). The involvement of these subunits in cancer adds an extra complexity to the VGCCs- cancer link mechanism.

TRP Calcium Permeable Channels

The Ca^{2+} permeable members of the TRP channel superfamily, which have a high diversity of gating mechanism (Nilius and Owsianik, 2010; Gees et al., 2012), are differently regulated in some cancer types. The remodeling of Ca^{2+} entrance and release pathways favors the phenotypic switch from normal to high proliferative state. For example, the expression of several TRP channels is elevated in different common carcinomas, e.g. TRPC1 in breast cancer (Dhennin-Duthille et al., 2011), TRPC3 in some breast and ovarian tumors (Aydar et al., 2009; Yang et al., 2009a; Tao et al., 2013), TRPC6 in breast, liver, stomach and glia cancers (El Boustany et al., 2008; Aydar et al., 2009; Cai et al., 2009; Ding et al., 2010; Dhennin-Duthille et al., 2011; Wen et al., 2016; Diez-Bello et al., 2019), TRPM7 in breast, pancreas, ovarian and gastric cancers (Hoshino et al., 1988; Kim et al., 2008; Kim et al., 2012; Middelbeek et al., 2012; Rybarczyk et al., 2012; Wang et al., 2014; Yee et al., 2015). TRPM8 is highly expressed at both mRNA and protein levels in tumor cells from different tissues: breast, pancreas, prostate, colorectal and lung (Schmidt et al., 2006; Chodon et al., 2010; Yee et al., 2010; Liu et al., 2014), but the expression is decreased during metastasis in prostate cancer cells via TCAF1 associated factor (Gkika et al., 2015). In this regard, a new pore-independent mechanism has been revealed, where TRPM8 inhibit endothelial cell migration via Rap1, a small GTPase, protein-protein interaction (Genova et al., 2017). TRPV6 expression levels are significantly high in different human carcinomas tissues, such as, breast, prostate, thyroid, colon and ovary (Wissenbach et al., 2001; Zhuang et al., 2002; Bolanz et al., 2008; Dhennin-Duthille et al., 2011). In contrast, the expression of other TRP channels appears to be decreased in cancer. For instance, TRPC4 channels in renal carcinoma (Veliceasa et al., 2007), TRPM6 in colorectal tumors (Xie et al., 2018), TRPV4 in skin and bladder tumor cells (Fusi et al., 2014; Mizuno et al., 2014; Yu et al., 2019) or the TRPV6 in lung carcinomas (Fan et al., 2014). In conclusion, the TRP channels superfamily expression levels have been described to be either up or down regulated in different tumors (**Table 1**). The specific molecular mechanism of these channels in cancer is not fully understood, but recent evidence suggests a key role for some of these channels in the cancer hallmarks (Villalobos et al., 2017; Prevarskaya et al., 2018). In addition, some members of the TRPC subfamily contribute to SOCE forming channel complexes with STIM1 and ORAI1 (Molnár et al., 2016; Villalobos et al., 2016). This set of proteins does not associate to form heterohexamers ORAI1/TRPC1/STIM1 complexes. Instead, ORAI1 and TRPC1 subunits form distinctive pore structures each responsible for its calcium selective permeability (Cheng et al., 2013). Further, TRPC1 activation is mediated by STIM1 binding, but TRPC1 trafficking to the membrane depends on ORAI1-based Ca^{2+} entry. Therefore, ORAI1/STIM1 and TRPC1/STIM1 assemble into two separate channels providing

TABLE 1 | Calcium permeable channels involved in different cancer types.

Ca^{2+} permeable channels		Cancer types	Expression profile	Reference
VGCCs	$Ca_V1.2$	Brain, breast, colorectal, pancreas, prostate, uterus, skin and esophageal	Gene upregulation	(Kale et al., 2015; Wang et al., 2015a; Cui et al., 2017; Anderson et al., 2019)
	$Ca_V1.3$	Breast, prostate, colorectal, bladder, gastric, uterus, lung, brain and esophageal	Gene and protein upregulation and gene downregulation	(Chen et al., 2014; Wang et al., 2015a; Phan et al., 2017; Prevarskaya et al., 2018)
	$Ca_V1.4$	Testis	Gene upregulation	(Wang et al., 2015a)
	$Ca_V2.1$	Leukemia, sarcoma, ovarian, brain, uterus, lung, cervix, colorectal, esophageal and gastric	Gene upregulation and downregulation	(Hao et al., 2006; Sabates-Bellver et al., 2007; Wang et al., 2015a; Phan et al., 2017)
	$Ca_V2.2$	Breast, brain and prostate	Gene up and downregulation	(Sun et al., 2006; Grasso et al., 2012; Wang et al., 2015a; Phan et al., 2017)
	$Ca_V3.1$	Breast, glioma, lung, prostate, colorectal, pancreas and gastric	Gene upregulation and downregulation	(Latour et al., 2004; Wang et al., 2015a; Cui et al., 2017; Anderson et al., 2019)
	$Ca_V3.2$	Prostate, renal, gastric, ovarian, brain, breast, bladder, lung, colon and skin	Gene and protein up and downregulation	(Gackière et al., 2008; Cui et al., 2017; Phan et al., 2017)
	$Ca_V3.3$	Breast, sarcoma, esophagus and gastric	Gene upregulation	(Wang et al., 2015a; Anderson et al., 2019)
TRPs	TRPC1	Breast, colorectal, esophageal, gastric and liver	Gene and protein upregulation	(Dhennin-Duthille et al., 2011; Villalobos et al., 2016; Cui et al., 2017; Anderson et al., 2019)
	TRPC3	Breast and ovarian	Gene and protein upregulation	(Aydar et al., 2009; Yang et al., 2009a; Tao et al., 2013)
	TRPC4	Renal	Gene downregulation	(Veliceasa et al., 2007)
	TRPC6	Breast, liver, stomach, prostate and glia	Gene and protein upregulation	(El Boustany et al., 2008; Guilbert et al., 2008; Aydar et al., 2009; Cai et al., 2009; Ding et al., 2010; Prevarskaya et al., 2018; Anderson et al., 2019)
	TRPM6	Colorectal	Gene downregulation	(Xie et al., 2018; Anderson et al., 2019)
	TRPM7	Breast, pancreas, ovarian, gastric and colorectal	Gene and protein upregulation	(Hoshino et al., 1988; Kim et al., 2008; Middelbeek et al., 2012; Rybarczyk et al., 2012; Wang et al., 2014; Bose et al., 2015; Anderson et al., 2019)
	TRPM8	Pancreas, prostate, bladder, skin, breast, colorectal, and lung	Gene and protein upregulation and gene downregulation	(Schmidt et al., 2006; Prevarskaya et al., 2007; Chodon et al., 2010; Yee et al., 2010; Liu et al., 2014; Cui et al., 2017; Anderson et al., 2019)
	TRPV4	Skin, renal and bladder	Gene and protein downregulation	(Fusi et al., 2014; Mizuno et al., 2014; Cui et al., 2017; Prevarskaya et al., 2018)
	TRPV6	Breast, prostate, thyroid, colon, lung and ovary	Gene and protein upregulation and gene downregulation	(Wissenbach et al., 2001; Zhuang et al., 2002; Bolanz et al., 2008; Dhennin-Duthille et al., 2011; Fan et al., 2014; Cui et al., 2017)
SOCs	ORAI1	Glia, breast, skin, colorectal, pancreas, esophageal, lung and renal	Gene and protein upregulation	(McAndrew et al., 2011; Motiani et al., 2013a; Kim et al., 2014; Zhu et al., 2014b; Cui et al., 2017; Anderson et al., 2019)
	ORAI3	Prostate, breast and lung	Gene and protein upregulation and gene downregulation	(Abeele et al., 2002; Ay et al., 2013; Motiani et al., 2013b; Dubois et al., 2014; Villalobos et al., 2016; Cui et al., 2017)
	STIM1	Glia, cervix, breast, lung, skin, liver, pancreas and colorectal	Gene and protein upregulation	(Scrideli et al., 2008; Chen et al., 2011; McAndrew et al., 2011; Li et al., 2013; Yang et al., 2013; Umemura et al., 2014; Wang et al., 2015b; Wang et al., 2016; Cui et al., 2017; Anderson et al., 2019)
	STIM2	Colorectal, skin, breast and glia	Gene upregulation and protein downregulation	(Ruano et al., 2006; Aytes et al., 2012; Sobradillo et al., 2014; Stanisz et al., 2014; Cui et al., 2017; Anderson et al., 2019)

individual calcium signals for regulation of different cell functions (Ambudkar et al., 2017; Villalobos et al., 2018). In fact, there is a long-term controversy on the function of TRPC channels in SOCE. The final consensus is that they may play a role in some cell types but not in others (Ambudkar et al., 2017). In anterior pituitary cells from mice lacking either ORAI1 or the seven TRPC1–7 channels, it was recently shown that SOCE required only ORAI1 channels but not any of the TRPC channels (Núñez et al., 2019). In human colonic cells, it seems that SOCE is mediated only by ORAI1 channels in normal cells. However, in colon cancer cells, SOCE depends on both ORAI1 and TRPC1 channels (Sobradillo et al., 2014) as reviewed next. Other member of the TRP channel superfamily, which is translocated to the plasma membrane via ORIA1-mediated mechanism, is the TRPV6 channel controlling the cancer cell survival (Raphaël et al., 2014).

Store Operated Calcium Channels

The mechanisms through SOCCs members contribute to cancer progression differ according to the molecular entity of the protein and the cancer type-specificity. It is important to remember that SOCCs are not just a single molecular unit, but rather multiprotein complex which includes PM and ER-membrane components, ORAI and STIM proteins respectively.

Although calcium signal remodeling involves changes of different Ca^{2+}-permeable channels, Ca^{2+} entry through ORAI channels is particularly important, as its dysregulation contribute to cancer features such as sustained proliferation, apoptosis resistance and invasion.

ORAI1 key component of SOCE, is essential for human brain tumor glioblastoma invasion. In these cells, the ORAI1 expression is upregulated causing an increased ORAI1-based calcium influx (Motiani et al., 2013a). In this regard, some breast cancer cell lines exhibit augmented levels of ORAI1, SOCE and the remodeling of the calcium influx associated with invasive stimuli (McAndrew et al., 2011; Zhu et al., 2014b). The ORAI1 involvement occurs also in melanoma cells, playing a central role in cell migration and proliferation, most likely via ERK signaling (Umemura et al., 2014). Furthermore, the ORAI1 interaction with SK3, a small conductance calcium-activated potassium channel, has been correlated with invasion and metastasis in colon and breast cancer, respectively, via SigmaR1 chaperone (Gueguinou et al., 2017). In renal carcinomas, the ORAI1 expression levels in cancer tissues were statistically higher than the levels in the adjacent normal parenchymal tissues, promoting cell proliferation and migration (Kim et al., 2014). These results suggest that, regardless the feasible differences in the cancer type-specificity and mechanism, ORAI1 plays a pivotal role in proliferation and invasion. However, this assumption is controversial because other authors propose that proliferation and apoptosis resistance is due to the ORAI3 remodeling. For instance, in prostate cancer cells, the overexpression of ORAI3 promotes two cancer hallmarks at once, enhanced proliferation and apoptosis resistance (Dubois et al., 2014). The ORAI3 increased expression levels favors the formation of ORAI1-ORAI3 heterohexamers over the ORAI1 homohexamers. The shift in the ORAI1/ORAI3 ratio reduces the Ca^{2+} influx from the extracellular space, promoting the enhanced apoptosis resistance shown by these cells (Abeele et al., 2002). These results highlight the essential role of ORAI1 in death related process (Flourakis et al., 2010). The downregulation of the ORAI1-based SOCE is associated with increased store independent calcium entry (SICE), promoting cancer cells proliferation via the Ca^{2+}/calcineurin-dependent transcription factor (Dubois et al., 2014). The ORAI3 expression is also elevated in breast cancer cells, where enhanced ORAI3-dependent SOCE result in apoptosis resistance, proliferation, and invasion in an estrogen receptor-dependent way (Motiani et al., 2013b). Association between elevated ORAI3 expression, enhanced ORAI3-based SOCE and high proliferation levels was also reported for non-small-cell lung adenocarcinoma (Ay et al., 2013) (**Table 1**).

The Ca^{2+} sensor STIM1 is overexpressed in different carcinomas, e.g., glioblastoma, cervix, breast, lung, liver, melanoma and colon (Scrideli et al., 2008; Chen et al., 2011; McAndrew et al., 2011; Li et al., 2013; Yang et al., 2013; Umemura et al., 2014; Wang et al., 2015b; Wang et al., 2016). In fact, STIM1 expression level is enhanced in early-stage cervical cancers, and using STIM1 siRNA, both SOCE and proliferation were inhibited in the cervical cancer cells. Furthermore, there is a positive correlation of local migration, tumor size and angiogenesis with STIM1 levels in cervical cancers cells (Chen et al., 2011). The abolition of STIM1 also prevents the cell proliferation and induces G0/G1 phase arrest of human glioblastoma cells (Li et al., 2013). In alveolar epithelial cells, STIM1 silencing attenuates the tumor growth, arresting cells in G1 phase in a p21 and cyclin D1-mediated pathway (Wang et al., 2016). In the same direction, STIM1 siRNA inhibits the migration and invasion abilities of the highly invasive tumor hepatic cells (Yang et al., 2013). Increased levels of STIM1 promotes growth and migration in colorectal cancer cells (CRC) and STIM1 elevated expression in CRC patients is associated with tumor size, invasion and metastasis (Wang et al., 2015b). Moreover, microarray analysis data show that, the basal-breast cancer, a breast cancer subtype with a very dismal prognosis, is correlated with an altered relationship between the ORAI1 activators STIM1 and STIM2, characterized by high STIM1/STIM2 ratios (McAndrew et al., 2011; Berna-Erro et al., 2017) (**Table 1**).

STIM1 homolog STIM2 senses smaller changes in ER-Ca^{2+} concentrations than STIM1, suggesting different cellular functions for these two Ca^{2+} sensors (Brandman et al., 2007). STIM1 detects deeper ER-Ca^{2+} depletions, due to its higher affinity for calcium, thus enabling the refilling of the depleted stores, while STIM2 detects lighter ER-Ca^{+2} depletions, keeping ER-Ca^{2+} concentrations within tight limits (Berna-Erro et al., 2017). Elevated STIM2 expression levels have been found in different cancer types, colorectal cancer (Aytes et al., 2012), human melanoma (Stanisz et al., 2014) and glioblastoma multiform tumors (Ruano et al., 2006). In colorectal cancer cells the high STIM2 expression levels has been related with a reduced invasive phenotype, suggesting a cancer cell growth suppressor function in contrast to STIM1 (Aytes et al., 2012; Berna-Erro et al., 2017). In the same vein, STIM2 overexpression was detected in human melanoma, where STIM2 siRNA treatment produced enhanced proliferation (Stanisz et al., 2014). These results correlate with the idea that, low STIM1/STIM2 ratios may be associated with less aggressive tumor conditions.

It is noteworthy that, while STIM2 gene expression levels are increased in CRC cells, STIM2 protein is practically absent in tumor cells, leading to the partial depletion of calcium stores (Sobradillo et al., 2014; Villalobos et al., 2016). The STIM2 loss in tumor cells moves internal Ca^{2+} store concentration near to the SOCE activation threshold and promotes apoptosis resistance and cell survival (Sobradillo et al., 2014). These discrepant results reveal the complexity role of STIM proteins in cancer and expand the classical function of controlling intracellular Ca^{2+} homeostasis. Additionally, the recent discovery of new STIM2 mRNA isoforms with opposite effects, might clarify these apparently contradictory reports. In these new reports, the novel variant STIM2.1 inhibits SOCE, whereas the former isoform STIM2.2 promotes it (Miederer et al., 2015) as detailed in (Berna-Erro et al., 2017).

INTRACELULAR CALCIUM HOMEOSTASIS

Intracellular Ca^{2+} concentrations control many different physiological and cellular functions at global and local levels, such as gene transcription in the nucleus, cell respiration and ATP synthesis in mitochondria or exocytosis in plasma membrane. To regulate this wide range of physiological Ca^{2+}-dependent processes, the cells maintain Ca^{2+} concentrations tightly controlled. This regulation is known as calcium homeostasis.

To keep calcium homeostasis and considering that Ca^{2+} is a cation and does not cross freely cell membranes, Ca^{2+} is transported through PM and internal membranes by particular transmembrane proteins, including Ca^{2+} permeable channels, Ca^{2+} pumps and ion transporters (Bong and Monteith, 2018; Villalobos et al., 2018). As we mentioned above, Ca^{2+} permeable channels enable Ca^{2+} entry into cells forced by the electrochemical gradient, formed by the difference in Ca^{2+} concentration between the internal (100 nM) and extracellular (1 mM) space and the membrane potential of about −60 mV. The calcium entry rises intracellular [Ca^{2+}] triggering different cell process. Then, it is removed from the cytosol and transported back to the extracellular medium or uptake into the intracellular Ca^{2+} stores by Ca^{2+} pumps and transporters, restoring the basal Ca^{2+} levels (Villalobos et al., 2018) (**Figure 1**).

This removing-Ca^{2+} process requires energy provided by the ATP hydrolysis for Ca^{2+}-pumps or the energy stored in the electrochemical gradient of other cation for transporters. For example, the Na^+/Ca^{2+} exchanger transports three Na^+ ions down its gradient in exchange of one Ca^{2+} cation against its electrochemical gradient (Reuter and Seitz, 1968). These dynamic Ca^{2+} signals differ with regard to their mechanisms of generation, spatial distributions and temporal properties, but are coordinated in space and time for a specific purpose. These calcium signals are characterized in different cell types and include calcium waves (Berridge et al., 2003; Parkash and Asotra, 2010), sparks (Cheng et al., 1993), calcium puffs (Yao et al., 1995), spikes (Meyer and Stryer, 1991), flickers (Wei et al., 2009), sparklets (Wang et al., 2001; Navedo et al., 2005; Tajada et al., 2017), etc. Compelling evidence show that variations in cellular Ca^{2+} dynamics participate in the normal and pathological signal transduction that controls cell proliferation and survival (Prevarskaya et al., 2018). Abnormal remodeling in Ca^{2+} homeostasis leads to the disruption of Ca^{2+} signaling resulting in a wide range of pathological conditions such as cell migration, excessive proliferation, invasion and programmed cell death resistance (Prevarskaya et al., 2011; Monteith et al., 2012; Prevarskaya et al., 2014).

Intracellular Ca^{2+}-storage organelles like the ER, mitochondria and the recently emerged as a major intracellular Ca^{2+} storage, lysosomes (Lloyd-Evans and Waller-Evans, 2019), also hold their own Ca^{2+} transporters. The ER is the largest single organelle and the most important intracellular Ca^{2+} store (Lam and Galione, 2013). It operates as a dynamic Ca^{2+} store thanks to the combined action of Ca^{2+} channels and transporters in the ER-membrane, and the Ca^{2+}-binding proteins in the ER-lumen, working as a high-capacity Ca^{2+} buffering system (Verkhratsky, 2005). Ca^{2+} is continuously leaking from the ER through leak channels still not identified. Nevertheless, Ca^{2+} concentration within the ER remains high due to the continuous activity of the endoplasmic reticulum Ca^{2+}-ATPases (SERCA) that replenish Ca^{2+} stores (MacLennan, 1970) (**Figure 1**). On the other hand, activation of the IP_3Rs, receptor-operated Ca^{2+} channels, release Ca^{2+} from intracellular store sites, contributing to the maintenance of a total amount of Ca^{2+} in the lumen similar to the extracellular space, in the mM range (Alvarez and Montero, 2002; Foskett et al., 2007). Because the lumen contains high concentrations of Ca^{+2} binding proteins (calreticulin, calsequestrin, annexin, calnexin, etc.) the concentration of free Ca^{+2} has been estimated to be between 100 and 700 μM (Foskett et al., 2007; Yáñez et al., 2012).

In the mitochondria, the Ca^{2+} uptake is conducted by the MCU and the Na^+/Ca^{2+} (NCLX) and Ca^{2+}/H^+ (HCX) exchangers are responsible for the mitochondrial Ca^{2+} release in exchange for Na^+ and H^+, respectively (Bononi et al., 2012; Srinivasan et al., 2015) (**Figure 1**). A large increase in Ca^{2+}, located near enough the MCU, triggers the influx of calcium to the mitochondrial matrix, rising the mitochondrial [Ca^{2+}] to very high concentrations near the mM level (Pozzan et al., 2000; Contreras et al., 2010). Mitochondria are also in dynamic contact with other organelles including the ER. These transient contacts between ER and mitochondria are essential to generate the highly localized and concentrated Ca^{2+} microdomains, facilitating the Ca^{2+} transport into mitochondria (Paupe and Prudent, 2018). Through this mitochondrial-ER crosstalk, IP_3Rs control the mitochondrial Ca^{2+} uptake and therefore metabolism and cell destiny (Cui et al., 2017). Although the mitochondria play an important role in Ca^{2+} homeostasis, the "*in vivo*" buffering capacity of this intracellular organelle is not particularly relevant (Pozzan et al., 2000).

Mitochondria not only modulate cytosolic Ca^{2+} signal. Mitochondrial calcium is also involved in the control of metabolism, ATP production and the regulation of cell death. Mitochondrial calcium regulates three important enzymes of the Krebs cycle: ketoglutarate, isocitrate, and pyruvate dehydrogenase. The effect of mitochondrial Ca^{2+} accumulation in Krebs cycle is the increase in the respiration rate and ATP synthesis (Mammucari et al., 2018). On the other hand, an excess of mitochondrial Ca^{2+} uptake, known as "mitochondrial calcium overload" triggers mitochondrial permeability transition pore (mPTP) opening. This is a high-conductance channel mediating mitochondrial swelling (Petronilli et al., 1993). This, together with other factors, triggers mitochondrial permeability transition, disruption of the mitochondrial potential and a massive release of cytochrome c, leading to the apoptosome activation and apoptosis (Giorgi et al., 2012; Mammucari et al., 2018).

The plasmalemma also displays many different calcium channels, ATP pumps and transporters to maintain the intracellular Ca^{2+} homeostasis. We have mentioned above the channels responsible of the Ca^{2+} influx. Now we will briefly describe the players involved in the transport of calcium out of

the cell to maintain the Ca^{2+} electrochemical gradient. Two players in the Ca^{2+} outflow from cells have been described, the Na^{+}/Ca^{2+} exchange transporter (NCX) and the plasma membrane Ca^{2+}-ATPase (PMCA) (**Figure 1**). The NCX is in charge of Ca^{2+} extrusion from the cytoplasm against its electrochemical gradient without ATP consumption, in fact, the electrochemical gradient of sodium stores that energy. In addition, NCX can operate in reverse mode contributing to Ca^{2+} entry in different conditions, such as cellular activation (Blaustein and Lederer, 1999). The PMCA pump with a 1:1 Ca^{2+}/ATP stoichiometry possesses a high-affinity but a low-capacity for Ca^{2+} transport, in total opposition to NCX (Noble and Herchuelz, 2007). This characteristic has conditioned the roles of these Ca^{2+} transporters. The PMCA performs a housekeeping role maintaining cytosolic Ca^{2+} around its basal level, whereas the NCX eliminates significant rises in intracellular Ca^{2+}, but this model has been revised during the latest years. See (Brini and Carafoli, 2011) for review.

CALCIUM PERMEABLE CHANNEL MODULATORS AND CANCER

Voltage-Gated Ca^{2+} Channel Modulators and Cancer

Several epidemiological investigations report that, calcium channel blockers used for the medical therapy of other pathologies are related with a reduced prostate cancer risk (Debes et al., 2004; Rodriguez et al., 2009), or at least a significantly tumor aggressiveness reduction (Poch et al., 2013). There is more discrepancy in studies of other cancer types, ranging from significant diminution in breast cancer risk (Fitzpatrick et al., 1997; Taylor et al., 2008) to no association between VGCCs blockers and cancer in colorectal, lung and breast tumors (Michels et al., 1998; Boudreau et al., 2008; Devore et al., 2015; Brasky et al., 2017; Bowles et al., 2019), including higher lung cancer risk correlated with exposure to calcium channels blockers (Rotshild et al., 2018).

Although, the potential benefit of the VGCCs blockers needs further preclinical studies, specific VGCCs antagonists, already approved by the FDA and EMA and used in clinical treatments, may be interesting for cancer treatments where individual VGCCs are overexpressed.

CRAC Channel Modulators and Cancer

The identification of CRAC channels modulators has been an important challenge for researchers and pharmaceuticals, since their discovery in 1992 (Hoth and Penner, 1992), because they have been implicated in numerous human pathologies, including pancreatitis, immunodeficiency, occlusive vascular diseases and cancer (Gerasimenko et al., 2013; Tian et al., 2016; Stauderman, 2018).

Numerous compounds have been described as inhibitors of SOCE or CRAC channels, which can be classified into four groups: inorganics, aptamers, antibodies and organic molecules (Stauderman, 2018). The inorganic lanthanides, specifically the trivalent cations, Lanthanum (La^{+3}) and Gadolinium (Gd^{+3}) are potent but non-selective blockers of the CRAC current (Parekh and Putney, 2005). Both can block directly the CRAC pore formed by the loopI-II of ORAI1, but also block other Ca^{2+} channels and pumps, such as VGCCs, TRPs and PMCA (Trebak et al., 2002; Yeromin et al., 2006; Cui et al., 2017). However, Gd^{3+} is normally used in Ca^{2+} imaging experiments to discriminate endogenous SOCs from other Ca^{2+} permeable channels such as recombinant TRPs, because the concentration that efficiently blocks the endogenous pathway does not interfere with the TRPs (Trebak et al., 2002; Parekh and Putney, 2005). In addition, these chemical elements are not viable for therapeutic treatment due to toxicological concerns. Aptamer Y1, an artificial single-stranded oligonucleotide engineered to bind with high affinity the first extracellular domain of the ORAI1 protein, block SOCE in the nM range in mast cells (Sun et al., 2016). However, the oligonucleotides present a disadvantage to be consider a potential drug candidate, the administration route is restricted to intravenous, subcutaneous or local delivery, as happened with the antibodies (Stauderman, 2018). Among antibodies, 10F8 (Cox et al., 2013), Anti-CRACM1-IgG (Liu et al., 2017), hHG1/LG1 (Komai et al., 2017) and mAb 2C1.1 (Lin et al., 2013) have been reported to partial block SOCE or CRAC currents due to the low specificity against all ORAI isoforms. Considering the organic small agents, the 2-aminoethoxydiphenylborane (2-APB), a membrane permeable antagonist of the IP_3-receptor activity, is widely used is SOCE experiments (Ma et al., 2000). Some studies reported that 2-APB inhibit cell proliferation and death resistance in gastric cancer cells (Sakakura et al., 2003; Cui et al., 2017) and cell migration in CRC cells (Abeele et al., 2002). Although, 2-APB is used as a reliable SOCE inhibitor (Bootman et al., 2002), it presents diverse effects on SOCE or CRAC currents. 2-APB is able to both, inhibit CRAC current without interference in the STIM1 and ORAI1 interaction (Yamashita et al., 2011) and directly activate ORAI3 channel independently of Ca^{2+} store depletion or STIM1 protein (Schindl et al., 2008). In addition, it presents a dose-dependent bimodal effect, stimulating CRAC currents at low concentration (less than 10 μM) and a transient increase followed by inhibition at higher (up to 50 μM) concentrations (Prakriya and Lewis, 2001). For this reason and because 2-APB modulates other proteins including SERCA (Missiaen et al., 2001), MagNum (Hermosura et al., 2002) and K^{+} channels (Wang et al., 2002), mitochondrial Ca^{2+} efflux (Prakriya and Lewis, 2001), gap junctions (Tao and Harris, 2007) and different TRP channels (Parekh and Putney, 2005; Derler et al., 2008), it should not be considered as a SOCE specific inhibitor (Parekh and Putney, 2005; Várnai et al., 2009). However, novel 2-APB derived compounds such as DBB-162AB and DPB-163AE with higher efficiency in SOCE inhibition and without unexpected interactions, are promising chemotherapeutic drugs (Goto et al., 2010).

Another class of small-molecules such as AnCoA4, ML-9, SB01990, KM06293 and RH01882 has been recently identified as SOCE inhibitors via different mechanism. AnCoA4 reduces the ORAI1 recruitment into puncta (Sadaghiani et al., 2014), ML-9

prevents STIM1 migration to ER-PM junctions (Smyth et al., 2008), SB01990, KM06293 and RH01882 modify the ORAI1 pore geometry, altering the Ca^{2+} selectivity and thereby reducing SOCE (Sampath and Sankaranarayanan, 2016; Cui et al., 2017).

Another compounds, imidazol, tenidap and SFK-96365 all classified as receptor-mediated Ca^{2+} entry blockers, were reported to inhibit CRAC currents in rat mast cells, but not in a specific manner (Franzius et al., 1994). For example, similar SKF96365 concentration used to block the CRAC currents, inhibits TRPC and TRPM channels with a comparable efficacy. The SKF-96365 was the first ORAI1 blocker used in cancer studies, specifically breast cancer. This imidazol derivative reduced tumor growth and invasion *in vivo* and inhibited cellular migration *in vitro* in breast cancer cells (Yang et al., 2009b). In other study, SKF-96365 significantly reduced tumor growth in esophageal cancer cells from immune deficient mouse, blocking ORAI-mediated SOCE and Ca^{2+} oscillations (Zhu et al., 2014a). Other compound targeting SOCE is the biomolecule Ohmline, a Edelfosine mimetic, that inhibits breast and colon cancer cells migration through the TRPC1/ORAI1/SK3 complex dissociation (Guéguinou et al., 2016).

Pyrazoles, heterocyclic organic compounds, specifically BTP1, BTP2 and BTP3, were identified by Abott and Astellas as SOCE blockers in Jurcat cells, using transcriptional and Ca^{2+} imaging assays (Djuric et al., 2000; Ishikawa et al., 2003; Stauderman, 2018). BTP2 (also known as Pyr2 or YM58483) is a pyrazole analogue that potently inhibits both TRPC-mediated and CRAC Ca^{2+} influx (He et al., 2005; Stauderman, 2018). However, similar to 2-APB and imidazole these compounds are not specific because they are involved in many other transport mechanisms. For instance, BTP2 also produces a PM depolarization via TRPM4 activation, therefore decreasing the Ca^{2+} driving force and consequently the Ca^{2+} influx inhibition (Takezawa et al., 2006). In contrast, other pyrazol-based compounds such as GSK-7975A (Derler et al., 2013), RO2959 (Chen et al., 2013) and the carboxamide GSK1349571A (Di Sabatino et al., 2009) seem to have reasonable selectivity with no affinity toward a wide range of receptors and ion channels and without affect TRPC1/5-mediated SOCE. Recently developed pyrazole analogues, such as, pyrazole-4-carboxamide (YW2065) and pyrimidine-2(1H)-thione derivative, have reported good anticancer activity in colorectal cancer cell lines compared to the standard drugs like sorafenib or oxaliplatin (Fahmy et al., 2016; Yang et al., 2019). More detailed information about the pharmacology of various CRAC channels modulators is revised in recent reports (Sweeney et al., 2009; Jairaman and Prakriya, 2013; Pevarello et al., 2014; Tian et al., 2016).

Other line of research is using small molecules compounds that modify the unbalance Ca^{2+} homeostasis in cancer cells. For example, the Nhr-BH4, a BCL-2 mimetic peptide that prevents the inhibition of the IP_3R Ca_{2+} release and thus unblock the apoptosis resistance in breast cancer cells (Nougarede et al., 2018). The TAT-IDPs, other BCL-2 based peptide, potentiates the pro-apoptotic Ca^{2+} signaling in chronic lymphocytic leukemia cells avoiding the IP_3R_2 inhibition, an IP_3R homolog, (Akl et al., 2013).

The importance of discovering new and specific inhibitors of CRAC channels and the development of new nanotechnology tools (Grolez et al., 2019), could be giant for the treatment of different human diseases. However, future pharmacology studies, and clinical trials will solve if these drugs are viable for therapeutic treatment.

ASPIRIN, NSAIDs AND POLYAMINE SYNTHESIS BLOCKERS AS CALCIUM CHANNEL MODULATORS IN CANCER

Overwhelming evidence provided in the last decades strongly suggest that acetylsalicylic acid (ASA), or aspirin provide protection against several forms of cancer, particularly those in the gastrointestinal tract (Chan et al., 2012; Rothwell et al., 2012; Drew et al., 2016). Same evidence has been also provided for a series of non-steroidal anti-inflammatory drugs (NSAIDs) like ibuprofen, flurbiprofen and sulindac, either alone or, in combination with other compounds like difluoromethylornithin (DFMO), a suicide inhibitor of polyamine biosynthesis (Tegeder et al., 2001; Rostom et al., 2007; Meyskens et al., 2008; Thompson et al., 2010; Chan et al., 2012; Dolejs et al., 2016). Evidence includes multiple data in cell lines and model animals, but also tens of epidemiological studies. Aspirin and NSAIDs inhibit several characteristic cancer cell hallmarks, including unrestrained cell proliferation, cell migration and invasion and cell death resistance. Numerous series of epidemiological evidences suggested that regular use of aspirin and/or several NSAIDs provide significant reduction in the risk of developing several forms of cancer and, particularly colorectal cancer. However, recommendation to use these over the counter drugs for cancer chemoprevention is hindered by the well-known secondary effects and/or risks of the regular use of NSAIDs leading to the conclusion that if, for any other reason or condition, individuals use NSAIDs regularly, they are probably protected against several forms of cancer. For any other situation, recommendation remains at hands of Doctors, that must evaluate whether the risk of taking NSAIDs is worthy in some cases of high risk of cancer. In fact, several studies have concluded that individuals at high risk of developing some forms of hereditary cancer like Lynch Syndrome may benefit of taking aspirin and/or NSAIDs to help protecting them from cancer (Burn et al., 2011; Yurgelun and Hampel, 2018). This may also apply to those patients that have received surgery for removing of polyps and/or tumors in the gastrointestinal tract that are also at high risk of recurrence. In these particular cases, the benefits are supported by several clinical trials that have addressed specifically the treatment and dose. For example, a clinical trial based in the combination of sulindac with DFMO in patients with previous tumors indicate that this combination may reduce nearly 90% the risk of developing recurrences (Meyskens et al., 2008; Sporn and Hong, 2008), revised in (Laukaitis and Gerner, 2011).

The action mechanisms of these drugs involved in preventing cancer cell hallmarks and cancer itself have been addressed in a large series of studies but they remain obscure. Most studies have

addressed the contribution of inflammation to carcinogenesis (Balkwill and Mantovani, 2001; Coussens and Werb, 2002) and the benefits of inhibiting inflammatory mechanisms in cancer cell (Hudes et al., 2013; Wu et al., 2013). However, although inflammation definitely contributes to cancer risk, several evidences suggest that additional and/or alternative mechanisms may contribute as well and some relevant mechanisms include modulation of the expression and/or activity of calcium permeable channels in cancer cells (Déliot and Constantin, 2015; Hoth, 2016; Prevarskaya et al., 2018).

Núñez et al. (2006) reported that salicylic acid, the most important metabolite of aspirin, inhibited store-operated Ca^{2+} entry (SOCE) and SOCE-dependent cell proliferation in colon cancer cells (Núñez et al., 2006; Valero et al., 2008). This effect cannot be attributed to inhibition of cyclooxygenase (COX) as salicylic acid lacks the acetyl moiety involved in COX acetylation, the mechanism involved in COX inhibition by aspirin. Also, it is achieved at concentrations far lower than the necessary to inhibit COX expression. Finally, the effects are similar in cells lacking COX2 expression implying that an alternative target is involved. On the other hand, same results are obtained by other NSAIDs including ibuprofen, indomethacin, sulindac and R-flubiprofen, the enantiomer unable to inhibit COX activity. Accordingly, salicylate and other NSAIDs inhibit SOCE and cell proliferation independently of COX activity and/or gene expression. Since SOCE is critical for cell proliferation, these drugs inhibit cell proliferation more likely because they inhibit SOCE rather than inflammatory pathways. This paradoxical view could be reconciled taken into account that SOCE may also contribute to inflammatory pathways as it is key in the activation of all cell types, including inflammatory cells. Importantly, inhibition of cell proliferation by salicylic acid is counteracted simply by enhancing extracellular Ca^{2+} just to restore Ca^{2+} entry in colon cancer cells. When taken together, these data indicate that salicylate (aspirin) and NSAIDs prevent cell proliferation in tumor and non-tumoral cells by inhibiting SOCE in a COX-independent manner.

In search for the mechanism of inhibition of SOCE by salicylate and other NSAIDs, Villalobos and cols. tested the effects of these drugs directly on Ca^{2+}-release activated currents (CRAC) responsible for SOCE in rat basophilic leukemia cells (RBL) and other cell types (Muñoz et al., 2011). These currents display a strong Ca^{2+}-dependent inactivation (Hoth et al., 1997; Fierro and Parekh, 2000) that render them unable to operate in a few seconds unless nearby mitochondria take up this calcium and sustain the current. In this sense, CRAC channels are dictated not only by the filling state of intracellular Ca^{2+} stores but also by mitochondria (Villalobos et al., 2018). Inhibition of the MCU, the calcium channel responsible of mitochondrial Ca^{2+} uptake, with ruthenium salts derivatives like RU386 or mitochondrial uncouplers like FCCP, that collapse the mitochondrial potential, the driving force for mitochondrial Ca^{2+} uptake, inhibit mitochondrial ability to take up Ca^{2+}, thus leading to Ca^{2+}-dependent inactivation of CRAC channels (Villalobos et al., 2019). Similar results have been obtained by MCU downregulation (Samanta et al., 2019). Salicylate and other NSAIDs are carboxylic acids bound to aromatic rings, the typical chemical structure of mitochondrial uncouplers. Accordingly, these drugs are able to decrease the mitochondrial potential or even collapsing it depending on concentration (Muñoz et al., 2011; Scatena, 2012; Hernández-Morales et al., 2017). As a consequence, at low concentrations they work as mild mitochondrial uncouplers that prevent mitochondrial Ca^{2+} uptake, leading to the Ca^{2+}-dependent inactivation of CRAC channels and SOCE inhibition in RBL cells. In conditions of high intracellular concentration of the Ca^{2+} buffer, when the Ca^{2+}-dependent inactivation cannot be achieved regardless of mitochondria, neither salicylate, NSAIDs nor MCU removal promote CRAC inactivation or SOCE inhibition. Therefore, salicylate and selected NSAIDs do not inhibit CRAC channels directly. Instead, these drugs promote the Ca^{2+}-dependent inactivation of these channels by preventing the Ca^{2+} uptake by mitochondria exactly as if we remove the MCU (Villalobos et al., 2019). These same results have been obtained also in other cell types as well, including vascular smooth muscle cells (VSMCs) (Muñoz et al., 2011; Muñoz et al., 2013) and colonic cells both normal and tumoral (Hernández-Morales et al., 2017).

Interestingly, the effects of salicylate and NSAIDs are achieved at very low concentrations in normal VSMCs leading to a full arrest of cell proliferation. VSMCs undergo phenotypic remodeling in different physiopathological situations. For example, upon damage, VSMCs may transform from differentiated, contractile cells to a proliferative and migrating phenotype that restores injury and returns to the differentiated phenotype. This process helps restoring damaged vessels but it may not be as beneficial if excessive proliferation occludes vessels as it happens in restenosis. This transdifferentation process is mediated by a change in the prevalence of different Ca^{2+}-permeable channels. In the differentiated phenotype, voltage-gated channels sensitive to dihydropyridines are prevalent. However, in the proliferative state, store-operated Ca^{2+} channels take the lead. These channels show strong Ca^{2+}-dependent inactivation that is prevented by mitochondria. Accordingly, low concentrations of salicylate and NSAIDs inhibit SOCE and cell proliferation in these cells during the proliferative phenotype but have no effect on Ca^{2+} entry during the differentiated phenotype. In fact, they have been proposed for treatment of restenosis, or excessive VSMC proliferation after stent implantation in coronary arteries (Weber et al., 2000; Koo et al., 2007; Dannoura et al., 2014).

As stated above, sensitivity of store-operated channels to aspirin and NSAIDs depends on mitochondria. As mitochondria from normal and tumor cells differ because of the Warburg effect, perhaps mitochondrial control of store-operated channels may be different as well in normal and colon cancer cells. Hernández-Morales et al. (2017) reported recently that this is indeed the case, at least in normal and colon cancer cells. Mitochondria from normal cells are not powerful enough to remove completely the Ca^{2+}-dependent inactivation of store-operated channels. However, in cancer cells, probably because of the Warburg effect, mitochondria display enhanced

mitochondrial potential and remove efficiently Ca^{2+}, thus preventing efficiently the Ca^{2+}-dependent inactivation of store-operated channels and sustaining Ca^{2+} entry (Hernández-Morales et al., 2017). Supporting this idea, the RNA expression analysis of genes involved in mitochondrial Ca^{2+} transport indicate that, MCU positive-modulator genes are overexpressed while negative-modulators are underexpressed in colon cancer cells (Pérez-Riesgo et al., 2017).

As stated above in the review, another difference between normal and colon cancer cells is that SOCE in normal colonic cells involves only ORAI1-mediated CRAC channels. In contrast, in colon cancer cells, SOCE is mediated by both ORAI1 and TRPC1 channels. Whereas ORAI1 channels in normal cells are essentially inactivating, in colon cancer cells, perhaps the involvement of TRPC1 channels may contribute to sustain currents. A possibility recently pointed out is that Na^+ influx mediated by TRPC1 channels may favor activity of the mitochondrial Na^+/Ca^{2+} exchanger, in turn preventing mitochondrial Ca^{2+} overload and the ensuing formation of reactive oxygen species (ROS) and ROS-dependent inactivation of ORAI1 channels (Quintana et al., 2011; Villalobos et al., 2018). This form of ROS-dependent regulation of ORAI1 channels remains controversial (Samanta et al., 2018; Samanta et al., 2019; Parekh, 2019).

In addition to aspirin and NSAIDs, other drugs have been reported to provide chemoprotection against several forms of cancer, particularly colorectal cancer. The most critical compound is DFMO, also called eflornithine. DFMO is considered a suicide inhibitor of ornithine decarboxylase 1 (ODC1), the critical enzyme in the biosynthesis pathway of the main mammalian polyamines: putrescine, spermine and spermidine. This enzyme is usually overexpressed in cancer cells, thus explaining the increased levels of polyamines that may contribute to cancer cell hallmarks, including enhanced cell proliferation, migration and invasion (Luk and Baylin, 1984; Pegg, 1988; Giardiello et al., 1997; Weiss et al., 2002). Several authors have reported ODC1 polymorphisms and robust correlation between specific polyamines and tissue growth (Martínez et al., 2003; Pegg, 2016; Wang et al., 2017; Sánchez-Jiménez et al., 2019). Finally, different growth stimuli including tumor promoters are able to enhance ODC activity and tumor formation (Bachmann and Geerts, 2018; Gerner et al., 2018). These effects are inhibited by DFMO, including inhibition of colon carcinogenesis in cancer models, such as the ApcMin/+ mice with high ODC levels and polyamines in the gastrointestinal tract. Interestingly, combinations of DFMO and NSAIDs have synergistic effects and inhibit dramatically carcinogenesis in both the small and large intestines of these mice. Recent clinical trials indicate that DFMO prevents CRC, especially if combined with sulindac, a carboxylic NSAID (Meyskens et al., 2008; Thompson et al., 2010; Burke et al., 2016).

Recent data reported by Gutierrez et al., suggest that DFMO may inhibit carcinogenesis by partially reversing the remodeling of store-operated Ca^{2+} channels in colorectal cancer cells (Gutiérrez et al., 2019). As stated above, colorectal cancers undergo a remodeling of intracellular Ca^{2+} homeostasis consisting in enhanced store-operated Ca^{2+} entry and decreased Ca^{2+} store content that contributed to cancer cell hallmarks, including enhanced cell proliferation, invasion and survival (Sobradillo et al., 2014; Villalobos et al., 2016; Villalobos et al., 2017). Villalobos and cols. have proposed that, during carcinogenesis, colorectal cancer cells may switch from a small, transient SOCE based on ORAI1-dependent CRAC channels (normal cells) to large and sustained currents depending on both ORAI1 and TRPC1 channels (tumor cells) (Villalobos et al., 2016; Villalobos et al., 2017; Villalobos et al., 2019). Interestingly, DFMO treatment inhibits cancer cell hallmarks in these cells including cell proliferation and cell death resistance. These effects are associated to SOCE inhibition and enhancement of Ca^{2+} store content. Therefore, DFMO may inhibit cancer cell hallmarks by reversing Ca^{2+} remodeling in colorectal cancer cells (Gutiérrez et al., 2019). Further research revealed that DFMO treatment inhibits TRPC1 expression and eliminates the TRPC1 component of the store-operated currents in colon cancer cells. These effects are reversed in the presence of polyamine putrescine (Gutiérrez et al., 2019). Collectively, these data suggest that polyamines promote Ca^{2+} channel remodeling by inducing expression of TRPC1 channels and this change promotes cancer cell hallmarks. In fact, this process may simply represent the exacerbation of the physiological process known as epithelial restitution that is mediated by transient polyamine synthesis induce by epithelial damage (Rao et al., 2012). While in physiological conditions this is a transient process limited by the repair of the wound epithelium, in cancer cells this process may became chronic due to ODC overexpression associated to activation of c-myc oncogene secondary to mutations in APC.

As stated above, combination of DFMO plus sulindac prevents efficiently colorectal cancer in high risk patients. An ongoing, large scale, three phase clinical trial is presently testing this treatment for patients at high risk of CRC (S0820, Adenoma and Second Primary Prevention Trial—Full Text View—ClinicalTrials.gov). Interestingly, Gutierrez et al. have reported that the combination of DFMO plus sulindac removes not only the TRPC1 component of the store-operated currents but also the ORAI1-dependent, thus fully abolishing the current. The mechanisms are different. The TRPC1-dependent current is removed at the expression level. However, the ORAI1-dependent current is simply inactivated because the mitochondria-dependent potentiation of the current is prevented. In summary, these data suggest a critical role for Ca^{2+} channel remodeling in cancer and provide a molecular mechanism of cancer chemoprevention targeting the remodeling of ion channels.

CONCLUDING REMARKS

It is clearly evident in the literature, that Ca^{2+}-permeable channels, transporters and pumps play important roles in a wide range of cancer-related process. The remodeling of these Ca^{2+}-permeable channels contributes to the Ca^{2+}-homeostasis dysregulation and both regulate several of the well-known cancer hallmarks. The influence of Ca^{2+} and other ion channels in carcinogenesis is so evident, that cancer was recently described as a channelopathy. This is a relative new field, however important advances in the identification of mechanism relating ion

channels and cancer has been done. For this reason, ion channels represent a novel and possibly important clinical targets for some cancer types, with only a few channel blockers tested in clinical trials.

The use of ion channels modulators as chemotherapeutic agents has their pros and cons. Most of the Ca^{2+}-permeable channels are ubiquitously expressed in different tissues, playing an important role in normal cell function. This is why, the pharmacological modulation of a specific cancer-related channel in tumor cells may produce significant toxicity in normal cells. Further research is required to take full advantage of channel modulators for cancer prevention, diagnosis and treatment.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to the writing of the manuscript.

FUNDING

This work has been supported by competitive grants RTI2018-099298-B-100 from Ministerio de Ciencia, Innovación y Universidades, Spain, BFU2015-70131 from Ministerio de Economía y Competitividad, Spain and VA294P18 from Junta de Castilla y León, Spain.

REFERENCES

Abeele, F., Vanden, Skryma, R., Shuba, Y., Van Coppenolle, F., Slomianny, C., Roudbaraki, M., et al. (2002). Bcl-2-dependent modulation of Ca2+ homeostasis and store-operated channels in prostate cancer cells. *Cancer Cell* 1, 169–179. doi: 10.1016/S1535-6108(02)00034-X

Akl, H., Monaco, G., La Rovere, R., Welkenhuyzen, K., Kiviluoto, S., Vervliet, T., et al. (2013). IP3R2 levels dictate the apoptotic sensitivity of diffuse large B-cell lymphoma cells to an IP3R-derived peptide targeting the BH4 domain of Bcl-2. *Cell Death Dis.* 4, 1–12. doi: 10.1038/cddis.2013.140

Alvarez, J., and Montero, M. (2002). Measuring [Ca2+] in the endoplasmic rerticulum with aequorin. *Cell Calcium* 32, 251–260. doi: 10.1016/S0143416002001860

Ambudkar, I. S., de Souza, L. B., and Ong, H. L. (2017). TRPC1, Orai1, and STIM1 in SOCE: Friends in tight spaces. *Cell Calcium* 63, 33–39. doi: 10.1016/j.ceca.2016.12.009

Anderson, K. J., Cormier, R. T., and Scott, P. M. (2019). Role of ion channels in gastrointestinal cancer. *World J. Gastroenterol.* 25 (38), 5732–5772. doi: 10.3748/wjg.v25.i38.5732

Ay, A.-S., Benzerdjerb, N., Sevestre, H., Ahidouch, A., and Ouadid-Ahidouch, H. (2013). Orai3 Constitutes a Native Store-Operated Calcium Entry That Regulates Non Small Cell Lung Adenocarcinoma Cell Proliferation. *PloS One.* 8 (9), 1–11. doi: 10.1371/journal.pone.0072889

Aydar, E., Yeo, S., Djamgoz, M., and Palmer, C. (2009). Abnormal expression, localization and interaction of canonical transient receptor potential ion channels in human breast cancer cell lines and tissues: A potential target for breast cancer diagnosis and therapy. *Cancer Cell Int.* 9, 1–12. doi: 10.1186/1475-2867-9-23

Aytes, A., Mollevi, D. G., Martinez-Iniesta, M., Nadal, M., Vidal, A., Morales, A., et al. (2012). Stromal interaction molecule 2 (STIM2) is frequently overexpressed in colorectal tumors and confers a tumor cell growth suppressor phenotype. *Mol. Carcinog.* 51, 746–753. doi: 10.1002/mc.20843

Bachmann, A. S., and Geerts, D. (2018). Polyamine synthesis as a target of MYC oncogenes. *J. Biol. Chem.* 293, 18757–18769. doi: 10.1074/jbc.TM118.003336

Badr, S. A., Fahmi, M. W., Nomir, M. M., and El-Shishtawy, M. M. (2018). Calcium channel α2δ1 subunit as a novel biomarker for diagnosis of hepatocellular carcinoma. *Cancer Biol. Med.* 15, 52–60. doi: 10.20892/j.issn.2095-3941.2017.0167

Balkwill, F., and Mantovani, A. (2001). Inflammation and cancer: Back to Virchow? *Lancet* 357, 539–545. doi: 10.1016/S0140-6736(00)04046-0

Batra, S., and Alenfall, J. (1991). Effect of diverse categories of drugs on human colon tumour cell proliferation. *Anticancer Res.* 11, 1221–1224.

Baughman, J. M., Perocchi, F., Girgis, H. S., Plovanich, M., Belcher-Timme, C. A., Sancak, Y., et al. (2011). Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. *Nature* 476, 341–345. doi: 10.1038/nature10234

Berchtold, M. W., and Villalobo, A. (2014). The many faces of calmodulin in cell proliferation, programmed cell death, autophagy, and cancer. Biochimica et Biophysica Acta - Molecular Cell Research 1843. *Biochim. Biophys. Acta - Mol. Cell Res.* 1843, 398–435. doi: 10.1016/j.bbamcr.2013.10.021

Berna-Erro, A., Jardin, I., Salido, G. M., and Rosado, J. A. (2017). Role of STIM2 in cell function and physiopathology. *J. Physiol.* 595, 3111–3128. doi: 10.1113/JP273889

Berridge, M. J., Bootman, M. D., and Roderick, H. L. (2003). Calcium signalling: Dynamics, homeostasis and remodelling. *Nat. Rev. Mol. Cell Biol.* 4, 517–529. doi: 10.1038/nrm1155

Berridge, M. J. (1983). Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol. *Biochem. J.* 212, 849–858. doi: 10.1042/bj2120849

Berridge, M. J. (1987). Inositol Trisphosphate and Diacylglycerol: Two Interacting Second Messengers. *Annu. Rev. Biochem.* 56, 159–193. doi: 10.1146/annurev.bi.56.070187.001111

Blaustein, M. P., and Lederer, W. J. (1999). Sodium/calcium exchange: Its physiological implications. *Physiol. Rev.* 79, 763–854. doi: 10.1152/physrev.1999.79.3.763

Bolanz, K. A., Hediger, M. A., and Landowski, C. P. (2008). The role of TRPV6 in breast carcinogenesis. *Mol. Cancer Ther.* 7, 271–279. doi: 10.1158/1535-7163.MCT-07-0478

Bong, A. H. L., and Monteith, G. R. (2018). Calcium signaling and the therapeutic targeting of cancer cells. *Biochim. Biophys. Acta - Mol. Cell Res.* 1865, 1786–1794. doi: 10.1016/j.bbamcr.2018.05.015

Bononi, A., Missiroli, S., Poletti, F., Suski, J. M., Agnoletto, C., Bonora, M., et al. (2012). Mitochondria-associated membranes (MAMs) as hotspot Ca(2+) signaling units. *Adv. Exp. Med. Biol.* 740, 411–437. doi: 10.1007/978-94-007-2888-2_17

Bootman, M. D., Collins, T. J., Mackenzie, L., Roderick, H. L., Berridge, M. J., and Peppiatt, C. M. (2002). 2-aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca2+ entry but an inconsistent inhibitor of InsP3-induced Ca2+ release. *FASEB J.* 16, 1145–1150. doi: 10.1096/fj.02-0037rev

Bose, T., Cieślar-Pobuda, A., and Wiechec, E. (2015). Role of ion channels in regulating Ca2+ homeostasis during the interplay between immune and cancer cells. *Cell Death Dis.* 6, 1–11. doi: 10.1038/cddis.2015.23

Boudreau, D. M., Koehler, E., Rulyak, S. J., Haneuse, S., Harrison, R., and Mandelson, M. T. (2008). Cardiovascular medication use and risk for colorectal cancer. *Cancer Epidemiol. Biomarkers Prev.* 17, 3076–3080. doi: 10.1158/1055-9965.EPI-08-0095

Bowles, E. J. A., Yu, O., Ziebell, R., Chen, L., Boudreau, D. M., Ritzwoller, D. P., et al. (2019). Cardiovascular medication use and risks of colon cancer recurrences and additional cancer events: A cohort study. *BMC Cancer* 19, 1–12. doi: 10.1186/s12885-019-5493-8

Brandman, O., Liou, J., Park, W. S., and Meyer, T. (2007). STIM2 Is a Feedback Regulator that Stabilizes Basal Cytosolic and Endoplasmic Reticulum Ca2+ Levels. *Cell* 131, 1327–1339. doi: 10.1016/j.cell.2007.11.039

Brasky, T. M., Krok-Schoen, J. L., Liu, J., Chlebowski, R. T., Freudenheim, J. L., Lavasani, S., et al. (2017). Use of calcium channel blockers and breast cancer risk in the women's health initiative. *Cancer Epidemiol. Biomarkers Prev.* 26, 1345–1348. doi: 10.1158/1055-9965.EPI-17-0096

Brini, M., and Carafoli, E. (2011). The Plasma Membrane Ca 2þ ATPase and the Plasma Membrane Sodium Calcium Exchanger Cooperate in the Regulation of Cell Calcium. *Cold Spring Harbor Perspect. Biol.* 3, 1–15. doi: 10.1101/cshperspect.a004168

Brücke, E.W.v. (1847). *De diffusione humorum per septa mortua et viva*. Ed. C. H. Schröder.

Buchanan, P. J., and McCloskey, K. D. (2016). CaV channels and cancer: canonical functions indicate benefits of repurposed drugs as cancer therapeutics. *Eur. Biophys. J.* 45, 621–633. doi: 10.1007/s00249-016-1144-z

Burke, C. A., Dekker, E., Samadder, N. J., Stoffel, E., and Cohen, A. (2016). Efficacy and safety of eflornithine (CPP-1X)/sulindac combination therapy versus each as monotherapy in patients with familial adenomatous polyposis (FAP): Design and rationale of a randomized, double-blind, Phase III trial. *BMC Gastroenterol.* 16, 87. doi: 10.1186/s12876-016-0494-4

Burn, J., Gerdes, A. M., MacRae, F., Mecklin, J. P., Moeslein, G., Olschwang, S., et al. (2011). Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: An analysis from the CAPP2 randomised controlled trial. *Lancet* 378, 2081–2087. doi: 10.1016/S0140-6736(11)61049-0

Cai, R., Ding, X., Zhou, K., Shi, Y., Ge, R., Ren, G., et al. (2009). Blockade of TRPC6 channels induced G2/M phase arrest and suppressed growth in human gastric cancer cells. *Int. J. Cancer* 125, 2281–2287. doi: 10.1002/ijc.24551

Chan, A. T., Arber, N., Burn, J., Chia, W. K., Elwood, P., Hull, M. A., et al. (2012). Aspirin in the chemoprevention of colorectal neoplasia: An overview. *Cancer Prev. Res.* 5, 164–178. doi: 10.1158/1940-6207.CAPR-11-0391

Chen, Y. F., Chiu, W. T., Chen, Y. T., Lin, P. Y., Huang, H. J., Chou, C. Y., et al. (2011). Calcium store sensor stromal-interaction molecule 1-dependent signaling plays an important role in cervical cancer growth, migration, and angiogenesis. *Proc. Natl. Acad. Sci. U. S. A.* 108, 15225–15230. doi: 10.1073/pnas.1103315108

Chen, G., Panicker, S., Lau, K. Y., Apparsundaram, S., Patel, V. A., Chen, S. L., et al. (2013). Characterization of a novel CRAC inhibitor that potently blocks human T cell activation and effector functions. *Mol. Immunol.* 54, 355–367. doi: 10.1016/j.molimm.2012.12.011

Chen, R., Zeng, X., Zhang, R., Huang, J., Kuang, X., Yang, J., et al. (2014). Cav1.3 channel α1D protein is overexpressed and modulates androgen receptor transactivation in prostate cancers. *Urol. Oncol. Semin. Orig. Investig.* 32, 524–536. doi: 10.1016/j.urolonc.2013.05.011

Cheng, H., Lederer, W. J., and Cannell, M. B. (1993). Calcium sparks: Elementary events underlying excitation-contraction coupling in heart muscle. *Sci. (80-.).* 262, 740–744. doi: 10.1126/science.8235594

Cheng, K. T., Ong, H. L., Liu, X., and Ambudkar, I. S. (2013). "Contribution and Regulation of TRPC Channels in Store-Operated Ca2+ Entry," in *Current Topics in Membranes* (Cambridge, Massachusetts, Estados Unidos: Academic Press Inc.), 149–179. doi: 10.1016/B978-0-12-407870-3.00007-X

Chodon, D., Guilbert, A., Dhennin-Duthille, I., Gautier, M., Telliez, M. S., Sevestre, H., et al. (2010). Estrogen regulation of TRPM8 expression in breast cancer cells. *BMC Cancer* 10, 1–8. doi: 10.1186/1471-2407-10-212

Contreras, L., Drago, I., Zampese, E., and Pozzan, T. (2010). Mitochondria: The calcium connection. *Biochim. Biophys. Acta - Bioenerg.* 1797, 607–618. doi: 10.1016/j.bbabio.2010.05.005

Coussens, L. M., and Werb, Z. (2002). Inflammation and cancer. *Nature* 420, 860–867. doi: 10.1038/nature01322

Cox, J. H., Hussell, S., Søndergaard, H., Roepstorff, K., Bui, J.-V., Deer, J. R., et al. (2013). Antibody-Mediated Targeting of the Orai1 Calcium Channel Inhibits T Cell Function. *PloS One* 8, e82944. doi: 10.1371/journal.pone.0082944

Cromer, M. K., Choi, M., Nelson-Williams, C., Fonseca, A. L., Kunstman, J. W., Korah, R. M., et al. (2015). Neomorphic effects of recurrent somatic mutations in yin yang 1 in insulin-producing adenomas. *Proc. Natl. Acad. Sci. U. S. A.* 112, 4062–4067. doi: 10.1073/pnas.1503696112

Cui, C., Merritt, R., Fu, L., and Pan, Z. (2017). Targeting calcium signaling in cancer therapy. *Acta Pharm. Sin. B* 7, 3–17. doi: 10.1016/j.apsb.2016.11.001

Déliot, N., and Constantin, B. (2015). Plasma membrane calcium channels in cancer: Alterations and consequences for cell proliferation and migration. *Biochim. Biophys. Acta - Biomembr.* 1848, 2512–2522. doi: 10.1016/j.bbamem.2015.06.009

Dannoura, A., Giraldo, A., Pereira, I., Gibbins, J. M., Dash, P. R., Bicknell, K. A., et al. (2014). Ibuprofen inhibits migration and proliferation of human coronary artery smooth muscle cells by inducing a differentiated phenotype: Role of peroxisome proliferator-activated receptor γ. *J. Pharm. Pharmacol.* 66, 779–792. doi: 10.1111/jphp.12203

Debes, J. D., Roberts, R. O., Jacobson, D. J., Girman, C. J., Lieber, M. M., Tindall, D. J., et al. (2004). Inverse Association between Prostate Cancer and the Use of Calcium Channel Blockers. *Cancer Epidemiol. Biomarkers Prev.* 13, 255–259. doi: 10.1158/1055-9965.EPI-03-0093

DeHaven, W.II, Smyth, J. T., Boyles, R. R., Bird, G. S., and Putney, J. W. (2008). Complex actions of 2-aminoethyldiphenyl borate on store-operated calcium entry. *J. Biol. Chem.* 283, 19265–19273. doi: 10.1074/jbc.M801535200

Derler, I., Fritsch, R., Schindl, R., and Romanin, C. (2008). CRAC inhibitors: Identification and potential. *Expert Opin. Drug Discovery* 3, 787–800. doi: 10.1517/17460441.3.7.787

Derler, I., Schindl, R., Fritsch, R., Heftberger, P., Riedl, M. C., Begg, M., et al. (2013). The action of selective CRAC channel blockers is affected by the Orai pore geometry. *Cell Calcium* 53, 139–151. doi: 10.1016/j.ceca.2012.11.005

Devore, E. E., Kim, S., Ramin, C. A., Wegrzyn, L. R., Massa, J., Holmes, M. D., et al. (2015). Antihypertensive medication use and incident breast cancer in women. *Breast Cancer Res. Treat.* 150, 219–229. doi: 10.1007/s10549-015-3311-9

Dhennin-Duthille, I., Gautier, M., Faouzi, M., Guilbert, A., Brevet, M., Vaudry, D., et al. (2011). High Expression of Transient Receptor Potential Channels in Human Breast Cancer Epithelial Cells and Tissues: Correlation with Pathological Parameters. *Cell. Physiol. Biochem.* 28, 813–822. doi: 10.1159/000335795

Di Sabatino, A., Rovedatti, L., Kaur, R., Spencer, J. P., Brown, J. T., Morisset, V. D., et al. (2009). Targeting Gut T Cell Ca 2+ Release-Activated Ca 2+ Channels Inhibits T Cell Cytokine Production and T-Box Transcription Factor T-Bet in Inflammatory Bowel Disease. *J. Immunol.* 183, 3454–3462. doi: 10.4049/jimmunol.0802887

Diez-Bello, R., Jardin, I., Lopez, J. J., El Haouari, M., Ortega-Vidal, J., Altarejos, J., et al. (2019). (–)□Oleocanthal inhibits proliferation and migration by modulating Ca2+ entry through TRPC6 in breast cancer cells. *Biochim. Biophys. Acta - Mol. Cell Res.* 1866, 474–485. doi: 10.1016/j.bbamcr.2018.10.010

Ding, X., He, Z., Zhou, K., Cheng, J., Yao, H., Lu, D., et al. (2010). Essential role of TRPC6 channels in G2/M phase transition and development of human glioma. *J. Natl. Cancer Inst.* 102, 1052–1068. doi: 10.1093/jnci/djq217

Dixon, R. E., Moreno, C. M., Yuan, C., Opitz-Araya, X., Binder, M. D., Navedo, M. F., et al. (2015). Graded Ca2+/calmodulin-dependent coupling of voltage-gated CaV1.2 channels. *Elife* 2015, 1–21. doi: 10.7554/eLife.05608

Djuric, S. W., Wiedeman, P. E., Zhou, X., Ballaron, S., Bauch, J., Chen, Y. W., et al. (2000). 3,5-Bis(trifluoromethyl)pyrazoles: A novel class of NFAT transcription factor regulator. *J. Med. Chem.* 43, 2975–2981. doi: 10.1021/jm990615a

Dolejs, S. C., Gayed, B., and Fajardo, A. (2016). Prevention of Colorectal Neoplasia. *Clin. Colon Rectal Surg.* 29, 353–362. doi: 10.1055/s-0036-1584086

Dolphin, A. C. (2016). Voltage-gated calcium channels and their auxiliary subunits: physiology and pathophysiology and pharmacology. *J. Physiol.* 594, 5369–5390. doi: 10.1113/JP272262

Drew, D. A., Cao, Y., and Chan, A. T. (2016). Aspirin and colorectal cancer: The promise of precision chemoprevention. *Nat. Rev. Cancer* 16, 173–186. doi: 10.1038/nrc.2016.4

Dubois, C., Vanden Abeele, F., Lehen'kyi, V., Gkika, D., Guarmit, B., Lepage, G., et al. (2014). Remodeling of Channel-Forming ORAI Proteins Determines an Oncogenic Switch in Prostate Cancer. *Cancer Cell* 26, 19–32. doi: 10.1016/j.ccr.2014.04.025

El Boustany, C., Bidaux, G., Enfissi, A., Delcourt, P., Prevarskaya, N., and Capiod, T. (2008). Capacitative calcium entry and transient receptor potential canonical 6 expression control human hepatoma cell proliferation. *Hepatology* 47, 2068–2077. doi: 10.1002/hep.22263

Endo, M., Tanaka, M., and Ogawa, Y. (1970). Calcium Induced release of calcium from the Sarcoplasmic Reticulum of Skinned Skeletal Muscle Fibres. *Nature* 228, 34–36. doi: 10.1038/228034a0

Fahmy, H., Khalifa, N., Ismail, M., El-Sahrawy, H., and Nossier, E. (2016). Biological Validation of Novel Polysubstituted Pyrazole Candidates with in Vitro Anticancer Activities. *Molecules* 21, 271. doi: 10.3390/molecules21030271

Fahrner, M., Schindl, R., Muik, M., Derler, I., and Romanin, C. (2017). "The STIM-orai pathway: The interactions between STIM and orai," in *Advances in*

Experimental Medicine and Biology (New York City (USA): Springer New York LLC), 59–81. doi: 10.1007/978-3-319-57732-6_4

Fan, H., Shen, Y. X., and Yuan, Y. F. (2014). Expression and prognostic roles of TRPV5 and TRPV6 in non-small cell lung cancer after curative resection. *Asian Pacific J. Cancer Prev.* 15, 2559–2563. doi: 10.7314/APJCP.2014.15.6.2559

Feske, S., Gwack, Y., Prakriya, M., Srikanth, S., Puppel, S. H., Tanasa, B., et al. (2006). A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature* 441, 179–185. doi: 10.1038/nature04702

Fierro, L., and Parekh, A. B. (2000). Substantial depletion of the intracellular Ca2+ stores is required for macroscopic activation of the Ca2+ release-activated Ca2 + current in rat basophilic leukaemia cells. *J. Physiol.* 522, 247–257. doi: 10.1111/j.1469-7793.2000.t01-1-00247.x

Fitzpatrick, A. L., Daling, J. R., Furberg, C. D., Kronmal, R. A., and Weissfeld, J. L. (1997). Use of calcium channel blockers and breast carcinoma risk in postmenopausal women. *Cancer* 80, 1438–1447. doi: 10.1002/(sici)1097-0142(19971015)80:8<1438::aid-cncr11>3.0.co;2-6

Fleischer, S., Ogunbunmi, E. M., Dixon, M. C., and Fleer, E. A. M. (1985). Localization of Ca2+ release channels with ryanodine in junctional terminal cisternae of sarcoplasmic reticulum of fast skeletal muscle. *Proc. Natl. Acad. Sci. U. S. A.* 82, 7256–7259. doi: 10.1073/pnas.82.21.7256

Flourakis, M., Lehen'kyi, V., Beck, B., Raphaël, M., Vandenberghe, M., Abeele, F. V., et al. (2010). Orai1 contributes to the establishment of an apoptosis-resistant phenotype in prostate cancer cells. *Cell Death Dis.* 1, 1–9. doi: 10.1038/cddis.2010.52

Foskett, J. K., White, C., Cheung, K. H., and Mak, D. O. D. (2007). Inositol trisphosphate receptor Ca2+ release channels. *Physiol. Rev.* 87, 593–658. doi: 10.1152/physrev.00035.2006

Franzius, D., Hoth, M., and Penner, R. (1994). Non-specific effects of calcium entry antagonists in mast cells. *Pflügers Arch. Eur. J. Physiol.* 428, 433–438. doi: 10.1007/BF00374562

Fukushima, M., Tomita, T., Janoshazi, A., and Putney, J. W. (2012). Alternative translation initiation gives rise to two isoforms of Orai1 with distinct plasma membrane mobilities. *J. Cell Sci.* 125, 4354–4361. doi: 10.1242/jcs.104919

Fusi, C., Materazzi, S., Minocci, D., Maio, V., Oranges, T., Massi, D., et al. (2014). Transient receptor potential vanilloid 4 (TRPV4) is downregulated in keratinocytes in human non-melanoma skin cancer. *J. Invest. Dermatol.* 134, 2408–2417. doi: 10.1038/jid.2014.145

Gackière, F., Bidaux, G., Delcourt, P., Van Coppenolle, F., Katsogiannou, M., Dewailly, E., et al. (2008). CaV3.2 T-type calcium channels are involved in calcium-dependent secretion of neuroendocrine prostate cancer cells. *J. Biol. Chem.* 283, 10162–10173. doi: 10.1074/jbc.M707159200

Gao, P., He, M., Zhang, C., and Geng, C. (2018). Integrated analysis of gene expression signatures associated with colon cancer from three datasets. *Gene* 654, 95–102. doi: 10.1016/j.gene.2018.02.007

Gees, M., Owsianik, G., Nilius, B., and Voets, T. (2012). TRP channels. *Compr. Physiol.* 2, 563–608. doi: 10.1002/cphy.c110026

Genova, T., Grolez, G. P., Camillo, C., Bernardini, M., Bokhobza, A., Richard, E., et al. (2017). TRPM8 inhibits endothelial cell migration via a nonchannel function by trapping the small GTPase Rap1. *J. Cell Biol.* 216, 2107–2130. doi: 10.1083/jcb.201506024

Gerasimenko, J. V., Gryshchenko, O., Ferdek, P. E., Stapleton, E., Hébert, T. O. G., Bychkova, S., et al. (2013). Ca2+ release-activated Ca2+ channel blockade as a potential tool in antipancreatitis therapy. *Proc. Natl. Acad. Sci. U. S. A.* 110, 13186–13191. doi: 10.1073/pnas.1300910110

Gerner, E. W., Bruckheimer, E., and Cohen, A. (2018). Cancer pharmacoprevention: Targeting polyamine metabolism to manage risk factors for colon cancer. *J. Biol. Chem.* 293, 18770–18778. doi: 10.1074/jbc.TM118.003343

Giardiello, F. M., Hamilton, S. R., Hylind, L. M., Yang, V. W., Tamez, P., and Casero, R. A. (1997). Ornithine decarboxylase and polyamines in familial adenomatous polyposis. *Cancer Res.* 57, 199–201.

Gilabert, J. A. (2000). Respiring mitochondria determine the pattern of activation and inactivation of the store-operated Ca2+ current ICRAC. *EMBO J.* 19, 6401–6407. doi: 10.1093/emboj/19.23.6401

Giorgi, C., Baldassari, F., Bononi, A., Bonora, M., De Marchi, E., Marchi, S., et al. (2012). Mitochondrial Ca2+ and apoptosis. *Cell Calcium* 52, 36–43. doi: 10.1016/j.ceca.2012.02.008

Gkika, D., Lemonnier, L., Shapovalov, G., Gordienko, D., Poux, C., Bernardini, M., et al. (2015). TRP channel-associated factors are a novel protein family that regulates TRPM8 trafficking and activity. *J. Cell Biol.* 208, 89–107. doi: 10.1083/jcb.201402076

Gomez-Ospina, N., Tsuruta, F., Barreto-Chang, O., Hu, L., and Dolmetsch, R. (2006). The C Terminus of the L-Type Voltage-Gated Calcium Channel CaV1.2 Encodes a Transcription Factor. *Cell* 127, 591–606. doi: 10.1016/j.cell.2006.10.017

Gomez-Ospina, N., Panagiotakos, G., Portmann, T., Pasca, S. P., Rabah, D., Budzillo, A., et al. (2013). A Promoter in the Coding Region of the Calcium Channel Gene CACNA1C Generates the Transcription Factor CCAT. *PloS One* 8, 1–11. doi: 10.1371/journal.pone.0060526

Goto, J.-I., Suzuki, A. Z., Ozaki, S., Matsumoto, N., Nakamura, T., Ebisui, E., et al. (2010). Two novel 2-aminoethyl diphenylborinate (2-APB) analogues differentially activate and inhibit store-operated Ca(2+) entry via STIM proteins. *Cell Calcium* 47, 1–10. doi: 10.1016/j.ceca.2009.10.004

Grasso, C. S., Wu, Y. M., Robinson, D. R., Cao, X., Dhanasekaran, S. M., Khan, A. P., et al. (2012). The mutational landscape of lethal castration-resistant prostate cancer. *Nature* 487, 239–243. doi: 10.1038/nature11125

Grolez, G. P., Hammadi, M., Barras, A., Gordienko, D., Slomianny, C., Völkel, P., et al. (2019). Encapsulation of a TRPM8 Agonist, WS12, in Lipid Nanocapsules Potentiates PC3 Prostate Cancer Cell Migration Inhibition through Channel Activation. *Sci. Rep.* 9, 1–15. doi: 10.1038/s41598-019-44452-4

Guéguinou, M., Harnois, T., Crottes, D., Uguen, A., Deliot, N., Gambade, A., et al. (2016). SK3/TRPC1/Orai1 complex regulates SOCE-dependent colon cancer cell migration: A novel opportunity to modulate anti- EGFR mAb action by the alkyl-lipid Ohmline. *Oncotarget* 7, 36168–36184. doi: 10.18632/oncotarget.8786

Gueguinou, M., Crottès, D., Chantôme, A., Rapetti-Mauss, R., Potier-Cartereau, M., Clarysse, L., et al. (2017). The SigmaR1 chaperone drives breast and colorectal cancer cell migration by tuning SK3-dependent Ca2+ homeostasis. *Oncogene* 36, 3640–3647. doi: 10.1038/onc.2016.501

Guilbert, A., Dhennin-Duthille, I., Hiani, Y. E. L., Haren, N., Khorsi, H., Sevestre, H., et al. (2008). Expression of TRPC6 channels in human epithelial breast cancer cells. *BMC Cancer* 8, 1–11. doi: 10.1186/1471-2407-8-125

Gutiérrez, L. G., Hernández-Morales, M., Núñez, L., and Villalobos, C. (2019). Inhibition of polyamine biosynthesis reverses Ca 2+ channel remodeling in colon cancer cells. *Cancers (Basel).* 11, 1–17. doi: 10.3390/cancers11010083

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. *Cell* 100, 57–70. doi: 10.1016/S0092-8674(00)81683-9

Hao, Y., Triadafilopoulos, G., Sahbaie, P., Young, H. S., Omary, M. B., and Lowe, A. W. (2006). Gene Expression Profiling Reveals Stromal Genes Expressed in Common Between Barrett's Esophagus and Adenocarcinoma. *Gastroenterology* 131, 925–933. doi: 10.1053/j.gastro.2006.04.026.

Haworth, A. S., and Brackenbury, W. J. (2019). Emerging roles for multifunctional ion channel auxiliary subunits in cancer. *Cell Calcium* 80, 125–140. doi: 10.1016/j.ceca.2019.04.005

He, L.-P., Hewavitharana, T., Soboloff, J., Spassova, M. A., and Gill, D. L. (2005). A functional link between store-operated and TRPC channels revealed by the 3,5-bis(trifluoromethyl)pyrazole derivative, BTP2. *J. Biol. Chem.* 280, 10997–11006. doi: 10.1074/jbc.M411797200

Hermosura, M. C., Monteilh-Zoller, M. K., Scharenberg, A. M., Penner, R., and Fleig, A. (2002). Dissociation of the store-operated calcium current/CRAC and the Mg-nucleotide-regulated metal ion current MagNuM. *J. Physiol.* 539, 445–458. doi: 10.1113/jphysiol.2001.013361

Hernández-Morales, M., Sobradillo, D., Valero, R. A., Muñoz, E., Ubierna, D., Moyer, M. P., et al. (2017). Mitochondria sustain store-operated currents in colon cancer cells but not in normal colonic cells: Reversal by non-steroidal anti-inflammatory drugs. *Oncotarget* 8, 55332–55352. doi: 10.18632/oncotarget.19430

Hoshino, Y., Obara, H., Kusunoki, M., Fujii, Y., and Iwai, S. (1988). Hypoxic contractile response in isolated human pulmonary artery: role of calcium ion. *J. Appl. Physiol.* 65, 2468–2474. doi: 10.1152/jappl.1988.65.6.2468

Hoth, M., and Penner, R. (1992). Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature* 355, 353–356. doi: 10.1038/355353a0

Hoth, M., Fanger, C. M., and Lewis, R. S. (1997). Mitochondrial regulation of store-operated calcium signaling in T lymphocytes. *J. Cell Biol.* 137, 633–648. doi: 10.1083/jcb.137.3.633

Hoth, M. (2016). CRAC channels, calcium, and cancer in light of the driver and passenger concept. *Biochim. Biophys. Acta - Mol. Cell Res.* 1863, 1408–1417. doi: 10.1016/j.bbamcr.2015.12.009

Huber, S. M. (2013). Oncochannels. *Cell Calcium* 53, 241–255. doi: 10.1016/j.ceca.2013.01.001

Hudes, G., Tagawa, S. T., Whang, Y. E., Qi, M., Qin, X., Puchalski, T. A., et al. (2013). A phase 1 study of a chimeric monoclonal antibody against interleukin-6, siltuximab, combined with docetaxel in patients with metastatic castration-resistant prostate cancer. *Invest. New Drugs* 31, 669–676. doi: 10.1007/s10637-012-9857-z

Ishikawa, J., Ohga, K., Yoshino, T., Takezawa, R., Ichikawa, A., Kubota, H., et al. (2003). A Pyrazole Derivative, YM-58483, Potently Inhibits Store-Operated Sustained Ca 2+ Influx and IL-2 Production in T Lymphocytes. *J. Immunol.* 170, 4441–4449. doi: 10.4049/jimmunol.170.9.4441

Jacquemet, G., Baghirov, H., Georgiadou, M., Sihto, H., Peuhu, E., Cettour-Janet, P., et al. (2016). L-type calcium channels regulate filopodia stability and cancer cell invasion downstream of integrin signalling. *Nat. Commun.* 7, 1–17. doi: 10.1038/ncomms13297

Jairaman, A., and Prakriya, M. (2013). Molecular pharmacology of store-operated CRAC channels. *Channels* 7, 402–414. doi: 10.4161/chan.25292

Kale, V. P., Amin, S. G., and Pandey, M. K. (2015). Targeting ion channels for cancer therapy by repurposing the approved drugs. *Biochim. Biophys. Acta - Biomembr.* 1848, 2747–2755. doi: 10.1016/j.bbamem.2015.03.034

Kim, B. J., Park, E. J., Lee, J. H., Jeon, J. H., Kim, S. J., and So, I. (2008). Suppression of transient receptor potential melastatin 7 channel induces cell death in gastric cancer. *Cancer Sci.* 99, 2502–2509. doi: 10.1111/j.1349-7006.2008.00982.x

Kim, B. J., Kim, S. Y., Lee, S., Jeon, J. H., Matsui, H., Kwon, Y. K., et al. (2012). The role of transient receptor potential channel blockers in human gastric cancer cell viability. *Can. J. Physiol. Pharmacol.* 90, 175–186. doi: 10.1139/Y11-114

Kim, J. H., Lkhagvadorj, S., Lee, M. R., Hwang, K. H., Chung, H. C., Jung, J. H., et al. (2014). Orai1 and STIM1 are critical for cell migration and proliferation of clear cell renal cell carcinoma. *Biochem. Biophys. Res. Commun.* 448, 76–82. doi: 10.1016/j.bbrc.2014.04.064

Komai, T., Kimura, T., Baba, D. Y., O.-U, P., and 2019, U. (2017). Anti-Orai1 antibody. US 2017/0226203.

Koo, B. K., Kim, Y. S., Park, K. W., Yang, H. M., Kwon, D. A., Chung, J. W., et al. (2007). Effect of celecoxib on restenosis after coronary angioplasty with a Taxus stent (COREA-TAXUS trial): an open-label randomised controlled study. *Lancet* 370, 567–574. doi: 10.1016/S0140-6736(07)61295-1

Kunzelmann, K. (2005). Ion channels and cancer. *J. Membr. Biol.* 205, 159–173. doi: 10.1007/s00232-005-0781-4

Lam, A. K. M., and Galione, A. (2013). The endoplasmic reticulum and junctional membrane communication during calcium signaling. *Biochim. Biophys. Acta - Mol. Cell Res.* 1833, 2542–2559. doi: 10.1016/j.bbamcr.2013.06.004

Latour, I., Louw, D. F., Beedle, A. M., Hamid, J., Sutherland, G. R., and Zamponi, G. W. (2004). Expression of T-type calcium channel splice variants in human glioma. *Glia* 48, 112–119. doi: 10.1002/glia.20063

Laukaitis, C. M., and Gerner, E. W. (2011). DFMO: Targeted risk reduction therapy for colorectal neoplasia. *Best Pract. Res. Clin. Gastroenterol.* 25, 495–506. doi: 10.1016/j.bpg.2011.09.007

Lee, S. C., Deutsch, C., and Beck, W. T. (1988). Comparison of ion channels in multidrug-resistant and -sensitive human leukemic cells. *Proc. Natl. Acad. Sci. U. S. A.* 85, 2019–2023. doi: 10.1073/pnas.85.6.2019

Li, G., Zhang, Z., Wang, R., Ma, W., Yang, Y., Wei, J., et al. (2013). Suppression of STIM1 inhibits human glioblastoma cell proliferation and induces G0/G1 phase arrest. *J. Exp. Clin. Cancer Res.* 32, 1–9. doi: 10.1186/1756-9966-32-20

Lin, F. F., Elliott, R., Colombero, A., Gaida, K., Kelley, L., Moksa, A., et al. (2013). Generation and characterization of fully human monoclonal antibodies against human orai1 for autoimmune diseases. *J. Pharmacol. Exp. Ther.* 345, 225–238. doi: 10.1124/jpet.112.202788

Liou, J., Kim, M. L., Won, D. H., Jones, J. T., Myers, J. W., Ferrell, J. E., et al. (2005). STIM is a Ca2+ sensor essential for Ca2+-store- depletion-triggered Ca2+ influx. *Curr. Biol.* 15, 1235–1241. doi: 10.1016/j.cub.2005.05.055

Lipscombe, D., Helton, T. D., and Xu, W. (2004). L-type calcium channels: The low down. *J. Neurophysiol.* 92, 2633–2641. doi: 10.1152/jn.00486.2004

Lis, A., Peinelt, C., Beck, A., Parvez, S., Monteilh-Zoller, M., Fleig, A., et al. (2007). CRACM1, CRACM2, and CRACM3 Are Store-Operated Ca2+ Channels with Distinct Functional Properties. *Curr. Biol.* 17, 794–800. doi: 10.1016/j.cub.2007.03.065

Liu, J., Chen, Y., Shuai, S., Ding, D., Li, R., and Luo, R. (2014). TRPM8 promotes aggressiveness of breast cancer cells by regulating EMT via activating AKT/GSK-3β pathway. *Tumor Biol.* 35, 8969–8977. doi: 10.1007/s13277-014-2077-8

Liu, S., Hasegawa, H., Takemasa, E., Suzuki, Y., Oka, K., Kiyoi, T., et al. (2017). Efficiency and Safety of CRAC Inhibitors in Human Rheumatoid Arthritis Xenograft Models. *J. Immunol.* 199, 1584–1595. doi: 10.4049/jimmunol.1700192

Lloyd-Evans, E., and Waller-Evans, H. (2019). Lysosomal Ca2+ Homeostasis and Signaling in Health and Disease. *Cold Spring Harb. Perspect. Biol.* 12, 1–12. doi: 10.1101/cshperspect.a035311

Lu, L., Sirish, P., Zhang, Z., Woltz, R. L., Li, N., Timofeyev, V., et al. (2015). Regulation of gene transcription by voltage-gated L-type calcium channel, Cav1.3. *J. Biol. Chem.* 290, 4663–4676. doi: 10.1074/jbc.M114.586883

Luk, G. D., and Baylin, S. B. (1984). Ornithine Decarboxylase as a Biologic Marker in Familial Colonic Polyposis. *N. Engl. J. Med.* 311, 80–83. doi: 10.1056/NEJM198407123110202

Ma, H. T., Patterson, R. L., Van Rossum, D. B., Birnbaumer, L., Mikoshiba, K., and Gill, D. L. (2000). Requirement of the inositol trisphosphate receptor for activation of store-operated Ca2+ channels. *Sci. (80-.).* 287, 1647–1651. doi: 10.1126/science.287.5458.1647

MacLennan, D. H. (1970). Purification and properties of an adenosine triphosphatase from sarcoplasmic reticulum. *J. Biol. Chem.* 245, 4508–4518.

Mammucari, C., Raffaello, A., Vecellio Reane, D., Gherardi, G., De Mario, A., and Rizzuto, R. (2018). Mitochondrial calcium uptake in organ physiology: from molecular mechanism to animal models. *Pflugers Archiv. Eur. J. Physiol.* 470, (8) 1165–1179. doi: 10.1007/s00424-018-2123-2

Mancini, M., and Toker, A. (2009). NFAT proteins: Emerging roles in cancer progression. *Nat. Rev. Cancer* 9, 810–820. doi: 10.1038/nrc2735

Martínez, M. E., O'Brien, T. G., Fultz, K. E., Babbar, N., Yerushalmi, H., Qu, N., et al. (2003). Pronounced reduction in adenoma recurrence associated with aspirin use and a polymorphism in the ornithine decarboxylase gene. *Proc. Natl. Acad. Sci. U. S. A.* 100, 7859–7864. doi: 10.1073/pnas.1332465100

McAndrew, D., Grice, D. M., Peters, A. A., Davis, F. M., Stewart, T., Rice, M., et al. (2011). ORAI1-mediated calcium influx in lactation and in breast cancer. *Mol. Cancer Ther.* 10, 448–460. doi: 10.1158/1535-7163.MCT-10-0923

Meyer, T., and Stryer, L. (1991). Calcium spiking. *Annu. Rev. Biophys. Biophys. Chem.* 20, 153–174. doi: 10.1146/annurev.bb.20.060191.001101

Meyskens, F. L., McLaren, C. E., Pelot, D., Fujikawa-Brooks, S., Carpenter, P. M., Hawk, E., et al. (2008). Difluoromethylornithine plus sulindac for the prevention of sporadic colorectal adenomas: A randomized placebo-controlled, double-blind trial. *Cancer Prev. Res.* 1, 32–38. doi: 10.1158/1940-6207.CAPR-08-0042

Michels, K. B., Rosner, B. A., Walker, A. M., Stampfer, M. J., Manson, J. E., Colditz, G. A., et al. (1998). Calcium channel blockers, cancer incidence, and cancer mortality in a cohort of U.S. Women: The nurses' health study. *Cancer* 83, 2003–2007. doi: 10.1002/(SICI)1097-0142(19981101)83:9<2003::AID-CNCR17>3.0.CO;2-3

Middelbeek, J., Kuipers, A. J., Henneman, L., Visser, D., Eidhof, I., Van Horssen, R., et al. (2012). TRPM7 is required for breast tumor cell metastasis. *Cancer Res.* 72, 4250–4261. doi: 10.1158/0008-5472.CAN-11-3863

Miederer, A. M., Alansary, D., Schwär, G., Lee, P. H., Jung, M., Helms, V., et al. (2015). A STIM2 splice variant negatively regulates store-operated calcium entry. *Nat. Commun.* 6, 1–12. doi: 10.1038/ncomms7899

Missiaen, L., Callewaert, G., De Smedt, H., and Parys, J. B. (2001). 2-Aminoethoxydiphenyl borate affects the inositol 1,4,5-trisphosphate receptor, the intracellular Ca2+ pump and the non-specific Ca2+ leak from the non-mitochondrial Ca2+ stores in permeabilized A7r5 cells. *Cell Calcium* 29, 111–116. doi: 10.1054/ceca.2000.0163

Mitra, R., Lee, J., Jo, J., Milani, M., McClintick, J. N., Edenberg, H. J., et al. (2011). Prediction of postoperative recurrence-free survival in non-small cell lung cancer by using an internationally validated gene expression model. *Clin. Cancer Res.* 17, 2934–2946. doi: 10.1158/1078-0432.CCR-10-1803

Mizuno, H., Suzuki, Y., Watanabe, M., Sokabe, T., Yamamoto, T., Hattori, R., et al. (2014). Potential role of transient receptor potential (TRP) channels in bladder cancer cells. *J. Physiol. Sci.* 64, 305–314. doi: 10.1007/s12576-014-0319-6

Molnár, T., Yarishkin, O., Iuso, A., Barabas, P., Jones, B., Marc, R. E., et al. (2016). Store-operated calcium entry in müller glia is controlled by synergistic activation of TRPC and orai channels. *J. Neurosci.* 36, 3184–3198. doi: 10.1523/JNEUROSCI.4069-15.2016

Monteith, G. R., Davis, F. M., and Roberts-Thomson, S. J. (2012). Calcium channels and pumps in cancer: changes and consequences. *J. Biol. Chem.* 287, 31666–31673. doi: 10.1074/jbc.R112.343061

Moreno, C. M., Dixon, R. E., Tajada, S., Yuan, C., Opitz-Araya, X., Binder, M. D., et al. (2016). Ca2+ entry into neurons is facilitated by cooperative gating of clustered Cav1.3 channels. *Elife* 5, 1–26. doi: 10.7554/eLife.15744

Motiani, R. K., Hyzinski-García, M. C., Zhang, X., Henkel, M. M., Abdullaev, I. F., Kuo, Y. H., et al. (2013a). STIM1 and Orai1 mediate CRAC channel activity and are essential for human glioblastoma invasion. *Pflugers Arch. Eur. J. Physiol.* 465, 1249–1260. doi: 10.1007/s00424-013-1254-8

Motiani, R. K., Zhang, X., Harmon, K. E., Keller, R. S., Matrougui, K., Bennett, J. A., et al. (2013b). Orai3 is an estrogen receptor α-regulated Ca^{2+} channel that promotes tumorigenesis. *FASEB J.* 27, 63–75. doi: 10.1096/fj.12-213801

Muñoz, E., Valero, R. A., Quintana, A., Hoth, M., Núñez, L., and Villalobos, C. (2011). Nonsteroidal anti-inflammatory drugs inhibit vascular smooth muscle cell proliferation by enabling the Ca2+-dependent inactivation of calcium release-activated calcium/orai channels normally prevented by mitochondria. *J. Biol. Chem.* 286, 16186–16196. doi: 10.1074/jbc.M110.198952

Muñoz, E., Hernández-Morales, M., Sobradillo, D., Rocher, A., Núñez, L., and Villalobos, C. (2013). Intracellular Ca2+ remodeling during the phenotypic journey of human coronary smooth muscle cells. *Cell Calcium* 54, 375–385. doi: 10.1016/j.ceca.2013.08.006

Núñez, L., Valero, R. A., Senovilla, L., Sanz-Blasco, S., García-Sancho, J., and Villalobos, C. (2006). Cell proliferation depends on mitochondrial Ca2+ uptake: inhibition by salicylate. *J. Physiol.* 571, 57–73. doi: 10.1113/jphysiol.2005.100586

Núñez, L., Bird, G. S., Hernando-Pérez, E., Pérez-Riesgo, E., Putney, J. W., and Villalobos, C. (2019). Store-operated Ca2+ entry and Ca2+ responses to hypothalamic releasing hormones in anterior pituitary cells from Orai1-/- and heptaTRPC knockout mice. *Biochim. Biophys. Acta Mol. Cell Res.* 1866, 1124–1136. doi: 10.1016/j.bbamcr.2018.11.006

Navedo, M. F., Amberg, G. C., Votaw, V. S., and Santana, L. F. (2005). Constitutively active L-type Ca2+ channels. *Proc. Natl. Acad. Sci.* 102, 11112–11117. doi: 10.1073/pnas.0500360102

Nilius, B., and Owsianik, G. (2010). Transient receptor potential channelopathies. *Pflugers Arch. Eur. J. Physiol.* 460, 437–450. doi: 10.1007/s00424-010-0788-2

Noble, D., and Herchuelz, A. (2007). meeting report Role of Na/Ca exchange and the plasma membrane Ca 2+-ATPase in cell function Conference on Na/Ca Exchange. *EMBO Rep.* 8, 228–232. doi: 10.1038/sj.embor.7400914

Nougarede, A., Popgeorgiev, N., Kassem, L., Omarjee, S., Borel, S., Mikaelian, I., et al. (2018). Breast cancer targeting through inhibition of the endoplasmic reticulum-based apoptosis regulator Nrh/BCL2L10. *Cancer Res.* 78, 1404–1417. doi: 10.1158/0008-5472.CAN-17-0846

Pardo, L. A. (2004). Voltage-gated potassium channels in cell proliferation. *Physiology* 19, 285–292. doi: 10.1152/physiol.00011.2004

Parekh, A. B., and Putney, J. W. (2005). Store-operated calcium channels. *Physiol. Rev.* 85, 757–810. doi: 10.1152/physrev.00057.2003

Parekh, A. B. (2019). Calcium signalling in health and disease. *Semin. Cell Dev. Biol.* 94, 1–2. doi: 10.1016/j.semcdb.2019.05.030

Parkash, J., and Asotra, K. (2010). Calcium wave signaling in cancer cells. *Life Sci.* 87, 587–595. doi: 10.1016/j.lfs.2010.09.013

Paupe, V., and Prudent, J. (2018). New insights into the role of mitochondrial calcium homeostasis in cell migration. *Biochem. Biophys. Res. Commun.* 500, 75–86. doi: 10.1016/j.bbrc.2017.05.039

Pegg, A. E. (1988). Polyamine Metabolism and Its Importance in Neoplastic Growth and as a Target for Chemotherapy. *Cancer Res.* 48, 759–774.

Pegg, A. E. (2016). Functions of polyamines in mammals. *J. Biol. Chem.* 291, 14904–14912. doi: 10.1074/jbc.R116.731661

Pérez-Riesgo, E., Gutiérrez, L. G., Ubierna, D., Acedo, A., Moyer, M. P., Núñez, L., et al. (2017). Transcriptomic Analysis of Calcium Remodeling in Colorectal Cancer. *Int. J. Mol. Sci.* 18, 1–23. doi: 10.3390/ijms18050922

Perocchi, F., Gohil, V. M., Girgis, H. S., Bao, X. R., McCombs, J. E., Palmer, A. E., et al. (2010). MICU1 encodes a mitochondrial EF hand protein required for Ca2+ uptake. *Nature* 467, 291–296. doi: 10.1038/nature09358

Petronilli, V., Cola, C., and Bernardi, P. (1993). Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore. II. The minimal requirements for pore induction underscore a key role for transmembrane electrical potential, matrix pH, and matrix Ca2+. *J. Biol. Chem.* 268, 1011–1016.

Pevarello, P., Cainarca, S., Liberati, C., Tarroni, P., Piscitelli, F., and Severi, E. (2014). Ca2+ release-activated Ca2+ channel inhibitors. *Pharm. Pat. Anal.* 3, 171–182. doi: 10.4155/ppa.14.7

Phan, N. N., Wang, C. Y., Chen, C. F., Sun, Z., Lai, M. D., and Lin, Y. C. (2017). Voltage-gated calcium channels: Novel targets for cancer therapy. *Oncol. Lett.* 14, 2059–2074. doi: 10.3892/ol.2017.6457

Platzer, J., Engel, J., Schrott-Fischer, A., Stephan, K., Bova, S., Chen, H., et al. (2000). Congenital deafness and sinoatrial node dysfunction in mice lacking class D L-type Ca2+ channels. *Cell* 102, 89–97. doi: 10.1016/S0092-8674(00)00013-1

Poch, M. A., Mehedint, D., Green, D. J., Payne-Ondracek, R., Fontham, E. T. H., Bensen, J. T., et al. (2013). The association between calcium channel blocker use and prostate cancer outcome. *Prostate* 73, 865–872. doi: 10.1002/pros.22632

Pozzan, T., Magalhães, P., and Rizzuto, R. (2000). The comeback of mitochondria to calcium signalling. *Cell Calcium* 28, 279–283. doi: 10.1054/ceca.2000.0166

Prakriya, M., and Lewis, R. S. (2001). Potentiation and inhibition of Ca(2+) release-activated Ca(2+) channels by 2-aminoethyldiphenyl borate (2-APB) occurs independently of IP(3) receptors. *J. Physiol.* 536, 3–19. doi: 10.1111/j.1469-7793.2001.t01-1-00003.x

Prevarskaya, N., Flourakis, M., Bidaux, G., Thebault, S., and Skryma, R. (2007). Differential role of TRP channels in prostate cancer. in. *Biochem. Soc. Trans.* 35, 133–135. doi: 10.1042/BST0350133

Prevarskaya, N., Skryma, R., and Shuba, Y. (2011). Calcium in tumour metastasis: new roles for known actors. *Nat. Rev. Cancer* 11, 609–618. doi: 10.1038/nrc3105

Prevarskaya, N., Ouadid-Ahidouch, H., Skryma, R., and Shuba, Y. (2014). Remodelling of Ca2+ transport in cancer: how it contributes to cancer hallmarks? *Philos. Trans. R. Soc Lond. B. Biol. Sci.* 369, 20130097. doi: 10.1098/rstb.2013.0097

Prevarskaya, N., Skryma, R., and Shuba, Y. (2018). Ion channels in cancer: Are cancer hallmarks oncochannelopathies? *Physiol. Rev.* 98, 559–621. doi: 10.1152/physrev.00044.2016

Putney, J. W. (2018). Forms and Functions of Store-operated Calcium Entry Mediators, STIM and Orai. *Adv. Bio. Regul.* 68, 88–96. doi: 10.1016/j.jbior.2017.11.006

Quintana, A., Pasche, M., Junker, C., Al-Ansary, D., Rieger, H., Kummerow, C., et al. (2011). Calcium microdomains at the immunological synapse: how ORAI channels, mitochondria and calcium pumps generate local calcium signals for efficient T-cell activation. *EMBO J.* 30, 3895–3912. doi: 10.1038/emboj.2011.289

Rao, J. N., Rathor, N., Zhuang, R., Zou, T., Liu, L., Xiao, L., et al. (2012). Polyamines regulate intestinal epithelial restitution through TRPC1-mediated Ca^2+ signaling by differentially modulating STIM1 and STIM2. *Am. J. Physiol. Cell Physiol.* 303, C308–C317. doi: 10.1152/ajpcell.00120.2012

Raphaël, M., Lehen'kyi, V., Vandenberghe, M., Beck, B., Khalimonchyk, S., Abeele, F. V., et al. (2014). TRPV6 calcium channel translocates to the plasma membrane via Orai1-mediated mechanism and controls cancer cell survival. *Proc. Natl. Acad. Sci. U. S. A.* 111, E3870–E3879. doi: 10.1073/pnas.1413409111

Reuter, H., and Seitz, N. (1968). The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. *J. Physiol.* 195, 451–470. doi: 10.1113/jphysiol.1968.sp008467

Rizzuto, R., Pinton, P., Carrington, W., Fay, F. S., Fogarty, K. E., Lifshitz, L. M., et al. (1998). Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca2+ responses. *Sci. (80-.).* 280, 1763–1766. doi: 10.1126/science.280.5370.1763

Rodriguez, C., Jacobs, E. J., Deka, A., Patel, A. V., Bain, E. B., Thun, M. J., et al. (2009). Use of blood-pressure-lowering medication and risk of prostate cancer in the Cancer Prevention Study II Nutrition Cohort. *Cancer Causes Control* 20, 671–679. doi: 10.1007/s10552-008-9280-0

Rostom, A., Dubé, C., Lewin, G., Tsertsvadze, A., Barrowman, N., Code, C., et al. (2007). Nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 inhibitors for primary prevention of colorectal cancer: a systematic review prepared for

the U.S. Preventive Services Task Force. *Ann. Intern. Med.* 146, 376–389. doi: 10.7326/0003-4819-146-5-200703060-00010

Rothwell, P. M., Price, J. F., Fowkes, F. G. R., Zanchetti, A., Roncaglioni, M. C., Tognoni, G., et al. (2012). Short-term effects of daily aspirin on cancer incidence, mortality, and non-vascular death: Analysis of the time course of risks and benefits in 51 randomised controlled trials. *Lancet* 379, 1602–1612. doi: 10.1016/S0140-6736(11)61720-0

Rotshild, V., Azoulay, L., Zarifeh, M., Masarwa, R., Hirsh-Raccah, B., Perlman, A., et al. (2018). The Risk for Lung Cancer Incidence with Calcium Channel Blockers: A Systematic Review and Meta-Analysis of Observational Studies. *Drug Saf.* 41, 555–564. doi: 10.1007/s40264-018-0644-4

Ruano, Y., Mollejo, M., Ribalta, T., Fiaño, C., Camacho, F.II, Gómez, E., et al. (2006). Identification of novel candidate target genes in amplicons of Glioblastoma multiforme tumors detected by expression and CGH microarray profiling. *Mol. Cancer* 5, 1–12. doi: 10.1186/1476-4598-5-39

Rybarczyk, P., Gautier, M., Hague, F., Dhennin-Duthille, I., Chatelain, D., Kerr-Conte, J., et al. (2012). Transient receptor potential melastatin-related 7 channel is overexpressed in human pancreatic ductal adenocarcinomas and regulates human pancreatic cancer cell migration. *Int. J. Cancer*. 131, E851–E861. doi: 10.1002/ijc.27487

Sánchez-Jiménez, F., Medina, M.Á, Villalobos-Rueda, L., and Urdiales, J. L. (2019). Polyamines in mammalian pathophysiology. *Cell. Mol. Life Sci.* 76, 3987–4008. doi: 10.1007/s00018-019-03196-0

S0820, Adenoma and Second Primary Prevention Trial - Full Text View - ClinicalTrials.gov.

Sabates-Bellver, J., Van Der Flier, L. G., De Palo, M., Cattaneo, E., Maake, C., Rehrauer, H., et al. (2007). Transcriptome profile of human colorectal adenomas. *Mol. Cancer Res.* 5, 1263–1275. doi: 10.1158/1541-7786.MCR-07-0267

Sadaghiani, A. M., Lee, S. M., Odegaard, J.II, Leveson-Gower, D. B., McPherson, O. M., Novick, P., et al. (2014). Identification of Orai1 channel inhibitors by using minimal functional domains to screen small molecule microarrays. *Chem. Biol.* 21, 1278–1292. doi: 10.1016/j.chembiol.2014.08.016

Sakakura, C., Hagiwara, A., Fukuda, K., Shimomura, K., Takagi, T., Kin, S., et al. (2003). Possible involvement of inositol 1,4,5-trisphosphate receptor type 3 (IP3R3) in the peritoneal dissemination of gastric cancers. *Anticancer Res.* 23, 3691–3697.

Samanta, K., Mirams, G. R., and Parekh, A. B. (2018). Sequential forward and reverse transport of the Na+ Ca2+ exchanger generates Ca2+ oscillations within mitochondria. *Nat. Commun.* 9, 1–10. doi: 10.1038/s41467-017-02638-2

Samanta, K., Bakowski, D., Amin, N., and Parekh, A. B. (2019). The whole-cell Ca 2+ release-activated Ca 2+ current, I CRAC , is regulated by the mitochondrial Ca 2+ uniporter channel and is independent of extracellular and cytosolic Na +. *J. Physiol.* 598, 1753–1773. doi: 10.1113/JP276551

Sampath, B., and Sankaranarayanan, K. (2016). Glu106 targeted inhibitors of ORAI1 as potential Ca2+ release-activated Ca2+ (CRAC) channel blockers - molecular modeling and docking studies. *J. Recept. Signal Transduction Res.* 36, 572–585. doi: 10.3109/10799893.2016.1141956

Sancak, Y., Markhard, A. L., Kitami, T., Kovács-Bogdán, E., Kamer, K. J., Udeshi, N. D., et al. (2013). EMRE is an essential component of the mitochondrial calcium uniporter complex. *Sci. (80-.).* 342, 1379–1382. doi: 10.1126/science.1242993

Scatena, R. (2012). Mitochondria and drugs. *Adv. Exp. Med. Biol.* 942, 329–346. doi: 10.1007/978-94-007-2869-1_15

Schindl, R., Bergsmann, J., Frischauf, I., Derler, I., Fahrner, M., Muik, M., et al. (2008). 2-aminoethoxydiphenyl borate alters selectivity of Orai3 channels by increasing their pore size. *J. Biol. Chem.* 283, 20261–20267. doi: 10.1074/jbc.M803101200

Schmidt, U., Fuessel, S., Koch, R., Baretton, G. B., Lohse, A., Tomasetti, S., et al. (2006). Quantitative multi-gene expression profiling of primary prostate cancer. *Prostate* 66, 1521–1534. doi: 10.1002/pros.20490

Schwab, A., Fabian, A., Hanley, P. J., and Stock, C. (2012). Role of ion channels and transporters in cell migration. *Physiol. Rev.* 92, 1865–1913. doi: 10.1152/physrev.00018.2011

Scrideli, C. A., Carlotti, C. G., Okamoto, O. K., Andrade, V. S., Cortez, M. A. A., Motta, F. J. N., et al. (2008). Gene expression profile analysis of primary glioblastomas and non-neoplastic brain tissue: Identification of potential target genes by oligonucleotide microarray and real-time quantitative PCR. *J. Neurooncol.* 88, 281–291. doi: 10.1007/s11060-008-9579-4

Secondo, A., Bagetta, G., and Amantea, D. (2018). On the role of store-operated calcium entry in acute and chronic neurodegenerative diseases. *Front. Mol. Neurosci.* 11, 1–14. doi: 10.3389/fnmol.2018.00087

Smyth, J. T., DeHaven, W.II, Bird, G. S., and Putney, J. W. (2008). Ca2+-store-dependent and -independent reversal of Stim1 localization and function. *J. Cell Sci.* 121, 762–772. doi: 10.1242/jcs.023903

Sobradillo, D., Hernández-Morales, M., Ubierna, D., Moyer, M. P., Núñez, L., and Villalobos, C. (2014). A reciprocal shift in transient receptor potential channel 1 (TRPC1) and stromal interaction molecule 2 (STIM2) contributes to Ca2+ remodeling and cancer hallmarks in colorectal carcinoma cells. *J. Biol. Chem.* 289, 28765–28782. doi: 10.1074/jbc.M114.581678

Sporn, M. B., and Hong, W. K. (2008). Concomitant DFMO and sulindac chemoprevention of colorectal adenomas: A major clinical advance. *Nat. Clin. Pract. Oncol.* 5, 628–629. doi: 10.1038/ncponc1221

Srinivasan, S., Chitalia, V., Meyer, R. D., Hartsough, E., Mehta, M., Harrold, I., et al. (2015). Hypoxia-induced expression of phosducin-like 3 regulates expression of VEGFR-2 and promotes angiogenesis. *Angiogenesis*. 18, 449–462. doi: 10.1007/s10456-015-9468-3

Stanisz, H., Saul, S., Müller, C. S. L., Kappl, R., Niemeyer, B. A., Vogt, T., et al. (2014). Inverse regulation of melanoma growth and migration by Orai1/STIM2-dependent calcium entry. *Pigment Cell Melanoma Res.* 27, 442–453. doi: 10.1111/pcmr.12222

Stauderman, K. A. (2018). CRAC channels as targets for drug discovery and development. *Cell Calcium* 74, 147–159. doi: 10.1016/j.ceca.2018.07.005

Streb, H., Irvine, R. F., Berridge, M. J., and Schulz, I. (1983). Release of Ca2+ from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature* 306, 67–69. doi: 10.1038/306067a0

Sun, L., Hui, A. M., Su, Q., Vortmeyer, A., Kotliarov, Y., Pastorino, S., et al. (2006). Neuronal and glioma-derived stem cell factor induces angiogenesis within the brain. *Cancer Cell* 9, 287–300. doi: 10.1016/j.ccr.2006.03.003

Sun, R., Yang, Y., Ran, X., and Yang, T. (2016). Calcium Influx of Mast Cells Is Inhibited by Aptamers Targeting the First Extracellular Domain of Orai1. *PloS One* 11, e0158223. doi: 10.1371/journal.pone.0158223

Sweeney, Z. K., Minatti, A., Button, D. C., and Patrick, S. (2009). Small-Molecule Inhibitors of Store-Operated Calcium Entry. *ChemMedChem* 4, 706–718. doi: 10.1002/cmdc.200800452

Tajada, S., Cidad, P., Colinas, O., Santana, L. F., López-López, J. R., and Pérez-García, M. T. (2013). Down-regulation of CaV1.2 channels during hypertension: How fewer CaV1.2 channels allow more Ca2+ into hypertensive arterial smooth muscle. *J. Physiol.* 591, 6175–6191. doi: 10.1113/jphysiol.2013.265751

Tajada, S., Moreno, C. M., O'Dwyer, S., Woods, S., Sato, D., Navedo, M. F., et al. (2017). Distance constraints on activation of TRPV4 channels by AKAP150-bound PKCα in arterial myocytes. *J. Gen. Physiol.* 149, 639–659. doi: 10.1085/jgp.201611709

Takezawa, R., Cheng, H., Beck, A., Ishikawa, J., Launay, P., Kubota, H., et al. (2006). A pyrazole derivative potently inhibits lymphocyte Ca2+ influx and cytokine production by facilitating transient receptor potential melastatin 4 channel activity. *Mol. Pharmacol.* 69, 1413–1420. doi: 10.1124/mol.105.021154

Tao, L., and Harris, A. L. (2007). 2-Aminoethoxydiphenyl borate directly inhibits channels composed of connexin26 and/or connexin32. *Mol. Pharmacol.* 71, 570–579. doi: 10.1124/mol.106.027508

Tao, X., Zhao, N., Jin, H., Zhang, Z., Liu, Y., Wu, J., et al. (2013). FSH enhances the proliferation of ovarian cancer cells by activating transient receptor potential channel C3. *Endocr. Relat. Cancer* 20, 415–429. doi: 10.1530/ERC-12-0005

Taylor, J. M., and Simpson, R. U. (1992). Inhibition of Cancer Cell Growth by Calcium Channel Antagonists in the Athymic Mouse. *Cancer Res.* 52, 2413–2418.

Taylor, J. T., Huang, L., Pottle, J. E., Liu, K., Yang, Y., Zeng, X., et al. (2008). Selective blockade of T-type Ca2+ channels suppresses human breast cancer cell proliferation. *Cancer Lett.* 267, 116–124. doi: 10.1016/j.canlet.2008.03.032

Tegeder, I., Pfeilschifter, J., and Geisslinger, G. (2001). Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *FASEB J.* 15, 2057–2072. doi: 10.1096/fj.01-0390rev

Thompson, P. A., Wertheim, B. C., Zell, J. A., Chen, W. P., McLaren, C. E., Lafleur, B. J., et al. (2010). Levels of rectal mucosal polyamines and prostaglandin E2

predict ability of DFMO and sulindac to prevent colorectal adenoma. *Gastroenterology* 139, 797–805, 805.e1. doi: 10.1053/j.gastro.2010.06.005

Tian, C., Du, L., Zhou, Y., and Li, M. (2016). Store-operated CRAC channel inhibitors: Opportunities and challenges. *Future Med. Chem.* 8, 817–832. doi: 10.4155/fmc-2016-0024

Trebak, M., Bird, G. S. J., McKay, R. R., and Putney, J. W. (2002). Comparison of human TRPC3 channels in receptor-activated and store-operated modes. Differential sensitivity to channel blockers suggests fundamental differences in channel composition. *J. Biol. Chem.* 277, 21617–21623. doi: 10.1074/jbc.M202549200

Umemura, M., Baljinnyam, E., Feske, S., De Lorenzo, M. S., Xie, L. H., Feng, X., et al. (2014). Store-operated Ca2+ entry (SOCE) regulates melanoma proliferation and cell migration. *PloS One* 9, 1–11. doi: 10.1371/journal.pone.0089292

Várnai, P., Hunyady, L., and Balla, T. (2009). STIM and Orai: the long-awaited constituents of store-operated calcium entry. *Trends Pharmacol. Sci.* 30, 118–128. doi: 10.1016/j.tips.2008.11.005

Valero, R. A., Senovilla, L., Núñez, L., and Villalobos, C. (2008). The role of mitochondrial potential in control of calcium signals involved in cell proliferation. *Cell Calcium*. 44, 259–269. doi: 10.1016/j.ceca.2007.12.002

Veliceasa, D., Ivanovic, M., Hoepfner, F. T.-S., Thumbikat, P., Volpert, O. V., and Smith, N. D. (2007). Transient potential receptor channel 4 controls thrombospondin-1 secretion and angiogenesis in renal cell carcinoma. *FEBS J.* 274, 6365–6377. doi: 10.1111/j.1742-4658.2007.06159.x

Verkhratsky, A. (2005). Physiology and pathophysiology of the calcium store in the endoplasmic reticulum of neurons. *Physiol. Rev.* 85, 201–279. doi: 10.1152/physrev.00004.2004

Villalobos, C., Núñez, L., Chamero, P., Alonso, M. T., and García-Sancho, J. (2001). Mitochondrial [Ca2+] Oscillations Driven by Local High [Ca 2+] Domains Generated by Spontaneous Electric Activity. *J. Biol. Chem.* 276, 40293–40297. doi: 10.1074/jbc.C100465200

Villalobos, C., Sobradillo, D., Hernández-Morales, M., and Núñez, L. (2016). "Remodeling of calcium entry pathways in cancer," in *Advances in Experimental Medicine and Biology* (New York City (USA): Springer New York LLC), 449–466. doi: 10.1007/978-3-319-26974-0_19

Villalobos, C., Sobradillo, D., Hernández-Morales, M., and Núñez, L. (2017). Calcium remodeling in colorectal cancer. *Biochim. Biophys. Acta - Mol. Cell Res.* 1864, 843–849. doi: 10.1016/j.bbamcr.2017.01.005

Villalobos, C., Gutiérrez, L. G., Hernández-Morales, M., del Bosque, D., and Núñez, L. (2018). Mitochondrial control of store-operated Ca 2+ channels in cancer: Pharmacological implications. *Pharmacol. Res.* 135, 136–143. doi: 10.1016/j.phrs.2018.08.001

Villalobos, C., Hernández-Morales, M., Gutiérrez, L. G., and Núñez, L. (2019). TRPC1 and ORAI1 channels in colon cancer. *Cell Calcium* 81, 59–66. doi: 10.1016/j.ceca.2019.06.003

Wang, S. Q., Song, L. S., Lakatta, E. G., and Cheng, H. (2001). Ca2+ signalling between single L-type Ca2+ channels and ryanodine receptors in heart cells. *Nature* 410, 592–596. doi: 10.1038/35069083

Wang, Y., Deshpande, M., and Payne, R. (2002). 2-Aminoethoxydiphenyl borate inhibits phototransduction and blocks voltage-gated potassium channels in Limulus ventral photoreceptors. *Cell Calcium* 32, 209–216. doi: 10.1016/S0143416002001562

Wang, J., Liao, Q. J., Zhang, Y., Zhou, H., Luo, C. H., Tang, J., et al. (2014). TRPM7 is required for ovarian cancer cell growth, migration and invasion. *Biochem. Biophys. Res. Commun.* 454, 547–553. doi: 10.1016/j.bbrc.2014.10.118

Wang, C. Y., Lai, M. D., Phan, N. N., Sun, Z., and Lin, Y. C. (2015a). Meta-analysis of public microarray datasets reveals voltage-gated calcium gene signatures in clinical cancer patients. *PloS One*. 10. doi: 10.1371/journal.pone.0125766

Wang, J. Y., Sun, J., Huang, M. Y., Wang, Y. S., Hou, M. F., Sun, Y., et al. (2015b). STIM1 overexpression promotes colorectal cancer progression, cell motility and COX-2 expression. *Oncogene* 34, 4358–4367. doi: 10.1038/onc.2014.366

Wang, Y., Wang, H., Li, L., Li, J., Pan, T., Zhang, D., et al. (2016). Elevated expression of STIM1 is involved in lung tumorigenesis. *Oncotarget* 7, 86584–86593. doi: 10.18632/oncotarget.13359

Wang, C., Ruan, P., Zhao, Y., Li, X., Wang, J., Wu, X., et al. (2017). Spermidine/spermine N1-acetyltransferase regulates cell growth and metastasis via AKT/β-catenin signaling pathways in hepatocellular and colorectal carcinoma cells. *Oncotarget* 8, 1092–1109. doi: 10.18632/oncotarget.13582

Wang, Z. (2004). Roles of K+ channels in regulating tumour cell proliferation and apoptosis. *Pflugers Arch. Eur. J. Physiol.* 448, 274–286. doi: 10.1007/s00424-004-1258-5

Warnier, M., Roudbaraki, M., Derouiche, S., Delcourt, P., Bokhobza, A., Prevarskaya, N., et al. (2015). CACNA2D2 promotes tumorigenesis by stimulating cell proliferation and angiogenesis. *Oncogene* 34, 5383–5394. doi: 10.1038/onc.2014.467

Weber, A. A., Yildirim, H., and Schrör, K. (2000). Cyclooxygenase-independent inhibition of smooth muscle cell mitogenesis by ibuprofen. *Eur. J. Pharmacol.* 389, 67–69. doi: 10.1016/S0014-2999(99)00868-7

Wei, C., Wang, X., Chen, M., Ouyang, K., Song, L. S., and Cheng, H. (2009). Calcium flickers steer cell migration. *Nature* 457, 901–905. doi: 10.1038/nature07577

Weiss, T. S., Bernhardt, G., Buschauer, A., Thasler, W. E., Dolger, D., Zirngibl, H., et al. (2002). Polyamine levels of human colorectal adenocarcinomas are correlated with tumor stage and grade. *Int. J. Colorectal Dis.* 17, 381–387. doi: 10.1007/s00384-002-0394-7

Wen, L., Liang, C., Chen, E., Chen, W., Liang, F., Zhi, X., et al. (2016). Regulation of Multi-drug Resistance in hepatocellular carcinoma cells is TRPC6/Calcium Dependent. *Sci. Rep.* 6, 1–14. doi: 10.1038/srep23269

Wissenbach, U., Niemeyer, B. A., Fixemer, T., Schneidewind, A., Trost, C., Cavalié, A., et al. (2001). Expression of CaT-like, a Novel Calcium-selective Channel, Correlates with the Malignancy of Prostate Cancer. *J. Biol. Chem.* 276, 19461–19468. doi: 10.1074/jbc.M009895200

Wu, C., Fernandez, S. A., Criswell, T., Chidiac, T. A., Guttridge, D., Villalona-Calero, M., et al. (2013). Disrupting cytokine signaling in pancreatic cancer: a phase I/II study of etanercept in combination with gemcitabine in patients with advanced disease. *Pancreas* 42, 813–818. doi: 10.1097/MPA.0b013e318279b87f

Wulff, H., Castle, N. A., and Pardo, L. A. (2009). Voltage-gated potassium channels as therapeutic targets. *Nat. Rev. Drug Discovery* 8, 982–1001. doi: 10.1038/nrd2983

Xiao, X., Li, B. X., Mitton, B., Ikeda, A., and Sakamoto, K. M. (2010). Targeting CREB for Cancer Therapy: Friend or Foe. *Curr. Cancer Drug Targets* 10, 384–391. doi: 10.2174/156800910791208535

Xie, B., Zhao, R., Bai, B., Wu, Y., Xu, Y., Lu, S., et al. (2018). Identification of key tumorigenesis-related genes and their microRNAs in colon cancer. *Oncol. Rep.* 40, 3551–3560. doi: 10.3892/or.2018.6726

Yáñez, M., Gil-Longo, J., and Campos-Toimil, M. (2012). Calcium binding proteins. *Adv. Exp. Med. Biol.* 740, 461–482. doi: 10.1007/978-94-007-2888-2_19

Yamashita, M., Somasundaram, A., and Prakriya, M. (2011). Competitive modulation of Ca2+ release-activated Ca2+ channel gating by STIM1 and 2-aminoethyldiphenyl borate. *J. Biol. Chem.* 286, 9429–9442. doi: 10.1074/jbc.M110.189035

Yang, S. L., Cao, Q., Zhou, K. C., Feng, Y. J., and Wang, Y. Z. (2009a). Transient receptor potential channel C3 contributes to the progression of human ovarian cancer. *Oncogene* 28, 1320–1328. doi: 10.1038/onc.2008.475

Yang, S., Zhang, J. J., and Huang, X. Y. (2009b). Orai1 and STIM1 Are Critical for Breast Tumor Cell Migration and Metastasis. *Cancer Cell* 15, 124–134. doi: 10.1016/j.ccr.2008.12.019

Yang, N., Tang, Y., Wang, F., Zhang, H., Xu, D., Shen, Y., et al. (2013). Blockade of store-operated Ca2+ entry inhibits hepatocarcinoma cell migration and invasion by regulating focal adhesion turnover. *Cancer Lett.* 330, 163–169. doi: 10.1016/j.canlet.2012.11.040

Yang, W., Li, Y., Ai, Y., Obianom, O. N., Guo, D., Yang, H., et al. (2019). Pyrazole-4-Carboxamide (YW2065): A Therapeutic Candidate for Colorectal Cancer via Dual Activities of Wnt/β-Catenin Signaling Inhibition and AMP-Activated Protein Kinase (AMPK) Activation. *J. Med. Chem.* 62, 11151–11164. doi: 10.1021/acs.jmedchem.9b01252

Yao, Y., Choi, J., and Parker, I. (1995). Quantal puffs of intracellular Ca2+ evoked by inositol trisphosphate in Xenopus oocytes. *J. Physiol.* 482, 533–553. doi: 10.1113/jphysiol.1995.sp020538

Yee, N. S., Zhou, W., and Lee, M. (2010). Transient receptor potential channel TRPM8 is over-expressed and required for cellular proliferation in pancreatic adenocarcinoma. *Cancer Lett.* 297, 49–55. doi: 10.1016/j.canlet.2010.04.023

Yee, N. S., Kazi, A. A., Li, Q., Yang, Z., Berg, A., and Yee, R. K. (2015). Aberrant over-expression of TRPM7 ion channels in pancreatic cancer: Required for

cancer cell invasion and implicated in tumor growth and metastasis. *Biol. Open*. 4, 507–514. doi: 10.1242/bio.20137088

Yeromin, A. V., Zhang, S. L., Jiang, W., Yu, Y., Safrina, O., and Cahalan, M. D. (2006). Molecular identification of the CRAC channel by altered ion selectivity in a mutant of Orai. *Nature* 443, 226–229. doi: 10.1038/nature05108

Yu, D., Holm, R., Goscinski, M. A., Trope, C. G., Nesland, J. M., and Suo, Z. (2016). Prognostic and clinicopathological significance of Cacna2d1 expression in epithelial ovarian cancers: A retrospective study. *Am. J. Cancer Res.* 6, 2088–2097.

Yu, S., Huang, S., Ding, Y., Wang, W., Wang, A., and Lu, Y. (2019). Transient receptor potential ion-channel subfamily V member 4: a potential target for cancer treatment. *Cell Death Dis.* 10, 1–17. doi: 10.1038/s41419-019-1708-9

Yurgelun, M. B., and Hampel, H. (2018). Recent Advances in Lynch Syndrome: Diagnosis, Treatment, and Cancer Prevention. *Am. Soc Clin. Oncol. Educ. B.* 38, 101–109. doi: 10.1200/edbk_208341

Zhu, H., Zhang, H., Jin, F., Fang, M., Huang, M., Yang, C. S., et al. (2014a). Elevated Orai1 expression mediates tumor-promoting intracellular Ca 2+ oscillations in human esophageal squamous cell carcinoma. *Oncotarget*. 5, 3455–3471. doi: 10.18632/oncotarget.1903

Zhu, M., Chen, L., Zhao, P., Zhou, H., Zhang, C., Yu, S., et al. (2014b). Store-operated Ca2+ entry regulates glioma cell migration and invasion via modulation of Pyk2 phosphorylation. *J. Exp. Clin. Cancer Res.* 33, 98. doi: 10.1186/s13046-014-0098-1

Zhuang, L., Peng, J., Tou, L., Takanaga, H., Adam, R. M., Hediger, M. A., et al. (2002). Calcium-selective ion channel, CaT1, is apically localized in gastrointestinal tract epithelia and is aberrantly expressed in human malignancies. *Lab. Investig.* 82, 1755–1764. doi: 10.1097/01.LAB.0000043910.41414.E7

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 Tajada and Villalobos. 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 Pharmacology

REVIEW
published: 23 July 2020
doi: 10.3389/fphar.2020.01111

The Functional Role of Voltage-Gated Sodium Channel Nav1.5 in Metastatic Breast Cancer

Qianxuan Luo[1,2†], Ting Wu[1,3†], Wenfang Wu[1], Gong Chen[1], Xuan Luo[4], Liping Jiang[3], Huai Tao[5], Mingqiang Rong[4], Shuntong Kang[1,3] and Meichun Deng[1,2]*

[1] Department of Biochemistry and Molecular Biology & Hunan Province Key Laboratory of Basic and Applied Hematology, School of Life Sciences, Central South University, Changsha, China, [2] Hunan Key Laboratory of Animal Models for Human Diseases & Hunan Key Laboratory of Medical Genetics, School of Life Sciences, Central South University, Changsha, China, [3] Xiangya School of Medicine, Central South University, Changsha, China, [4] Department of Biochemistry and Molecular Biology, Hunan Normal University, Changsha, China, [5] Department of Biochemistry and Molecular Biology, Hunan University of Chinese Medicine, Changsha, China

OPEN ACCESS

Edited by:
Lin-Hua Jiang, University of Leeds, United Kingdom

Reviewed by:
Mustafa Bilgin Djamgoz, Imperial College London, United Kingdom
Sébastien Roger, Université de Tours, France

***Correspondence:**
Meichun Deng
dengmch@csu.edu.cn

[†]*These authors have contributed equally to this work*

Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *17 January 2020*
Accepted: *08 July 2020*
Published: *23 July 2020*

Citation:
Luo Q, Wu T, Wu W, Chen G, Luo X, Jiang L, Tao H, Rong M, Kang S and Deng M (2020) The Functional Role of Voltage-Gated Sodium Channel Nav1.5 in Metastatic Breast Cancer. Front. Pharmacol. 11:1111. doi: 10.3389/fphar.2020.01111

Voltage-gated sodium channels (VGSCs), which are abnormally expressed in various types of cancers such as breast cancer, prostate cancer, lung cancer, and cervical cancer, are involved in the metastatic process of invasion and migration. Nav1.5 is a pore-forming α subunit of VGSC encoded by *SCN5A*. Various studies have demonstrated that Nav1.5, often as its neonatal splice form, is highly expressed in metastatic breast cancer cells. Abnormal activation and expression of Nav1.5 trigger a variety of cellular mechanisms, including changing H^+ efflux, promoting epithelial-to-mesenchymal transition (EMT) and the expression of cysteine cathepsin, to potentiate the metastasis and invasiveness of breast cancer cells *in vitro* and *in vivo*. Here, we systematically review the latest available data on the pro-metastatic effect of Nav1.5 and its underlying mechanisms in breast cancer. We summarize the factors affecting Nav1.5 expression in breast cancer cells, and discuss the potential of Nav1.5 blockers serving as candidates for breast cancer treatment.

Keywords: VGSCs, Nav1.5, breast cancer, metastasis, mechanism, inhibitors

INTRODUCTION

Breast cancer accounts for the largest proportion of women's mortality worldwide (Greaney et al., 2015). In the United States, approximately 12% of women will develop invasive breast cancer during their lifetime. The diagnosis of at least 276,480 new cases of invasive breast cancer and 42,170 breast cancer associated deaths are estimated in 2020 (Breastcancer.org). It is a complicated disease with inter- and intra-tumoral heterogeneity, and there are several different classifications of breast cancer according to advanced technical methods, including histological stratification and gene expression clustering (Sorlie et al., 2001; Margan et al., 2016; Yeo and Guan, 2017). The different molecular types of breast cancer correspond to different molecular mechanisms and require specific therapeutic strategies (Prat et al., 2015; Arciero et al., 2019). Therefore, appropriate management and therapies are necessary to achieve the best response to improve the prognosis and survival of patients. Notably, breast cancer cells have the potential to metastasize to distant organs such as the

brain, lung, liver, and bone (Hoshino et al., 2015; Hurvitz et al., 2018; Park et al., 2018; Zhu et al., 2019). Cancer metastasis is a complex, multi-step process that is closely associated with cells' local invasion, blood and lymphatic diffusion, and extravasation and colonization at distant sites. Metastatic cancer cells degrade the extracellular matrix, detach from their original location, and enter the circulation system through which they reach and colonize distant organs (Mego et al., 2010; Massague et al., 2017). Metastasis is the direct cause of death from cancer, accounting for nearly 90% of deaths related to breast cancer (Xie et al., 2017). Thus, there is an urgent need to identify the underlying molecular mechanisms of breast cancer and develop effective therapeutic strategies.

Voltage-gated sodium channels (VGSCs) are primarily expressed in excitable cells such as neurons. Na^+ influx regulated by VGSCs produces action potential and propagates excitability. VGSCs also exist in non-excitable cells, including microglia, astrocytes, immune cells, fibroblasts, and cancer cells, where they regulate and influence an array of biological functions such as phagocytosis, motility, Na^+/K^+-ATPase activity, and metastatic activity (Black and Waxman, 2013). VGSCs are composed of pore-forming α subunits and auxiliary β subunits (Hull and Isom, 2018). The family Nav1 comprises nine genes (*SCN1A-5A*, *SCN8A-11A*) that encode α subunits, Nav1.1 to Nav1.9. Each α subunit comprises four homologous domains, and each domain consists of six transmembrane segments (Catterall, 2000). Five β subunits, including β1 and its splice variants β1B and β2–β4, are encoded by four genes (*SCN1B-SCN4B*) (Brackenbury and Isom, 2011). These β subunits have been found to modulate the bioelectrical characteristics of α subunits and function as cellular adhesion molecules. Both α and β subunits play a critical role in the development of the central nervous system (CNS), and altering the expression of specific subunits may cause developmental aberrations and CNS lesions. Furthermore, expression of α and β subunits is upregulated in various cancers such as breast cancer, prostate cancer, lung cancer, and cervical cancer, observed both in *in vitro* and *in vivo* systems. They are found to enhance metastasis progression, including invasion, migration, endocytosis, and gene expression, *via* the Na^+ currents carried by α subunits and the adhesion interaction regulated by β subunits (Yang et al., 2012). The correlation between VGSCs-inhibiting drug uses and outcomes of cancer patients has been highlighted recently (Fairhurst et al., 2015; Reddy et al., 2015; Kao et al., 2018), even though the conclusions are still inconsistent. Hence, randomized controlled trials are required to exclude confounding factors and obtain a convincing conclusion in the future.

Nav1.5, a pore-forming α subunit of VGSC encoded by *SCN5A*, is expressed in lymphoma, neuroblastoma, breast and prostate cancer cells. Aberrant expression and activity of Nav1.5 are associated with cardiac excitability diseases such as arrhythmic dilated cardiomyopathy, Brugada syndrome, and long QT syndrome (Antzelevitch et al., 2005; Amin et al., 2013; Giudicessi and Ackerman, 2013; Beckermann et al., 2014). Nav1.5 is also overexpressed in metastatic breast cancer *in vitro* and *in vivo* and mainly exists in its DI: S3 5′ neonatal splice form (nNav1.5) in breast cancer cells, potentiating cell metastasis (Mohammed et al., 2016; Yamaci et al., 2017). Breast cancer cells treated with specific Nav1.5 inhibitors or siRNAs show decreased motility and metastatic capacity (Driffort et al., 2014).Therefore, Nav1.5 may be regarded as a promising target for the diagnosis and therapy of breast cancer.

With the high motility and metastatic capacity of breast cancer, it is imperative to determine the mechanisms of pro-metastatic effects of Nav1.5 and develop effective Nav1.5 inhibitors for breast cancer treatment. This review clarifies the role and mechanisms of Nav1.5 in metastatic breast cancer progression and summarizes some drugs with remarkable effects on reducing metastasis of breast cancer by acting on Nav1.5. All these evidence supports the idea that Nav1.5 as an anti-metastatic target for the treatment of metastatic breast cancer.

Nav1.5 Expression and Its Functional Role in Breast Cancer Metastasis

Nav1.5 in its neonatal DI:S3 5′ splice form is predominantly expressed in metastatic cancer cells (Fraser et al., 2005; Yamaci et al., 2017). This form has been found to participate in neonatal development, while it is absent in postnatal development. The overexpression of Nav1.5 in cancer cells suggests that embryonic genes are re-expressed during ontogenesis and participate in many cellular behaviors related to metastasis (Monk and Holding, 2001).

The expression levels of Nav1.5 and nNav1.5 in the highly metastatic MDA-MB-231 breast cancer cell line were significantly higher than those in weakly metastatic MCF-7 cells (Kamarulzaman et al., 2017; Zhang et al., 2018). Nav1.5 is specifically present on the membrane of MDA-MB-231 cells, but not in normal cell lines and weakly metastatic MCF-7 cells. In breast cancers, Nav1.5 α subunit mRNA and protein expression correlates with metastatic potential, and the neonatal *SCN5A* splice variant is expressed ~1,800-fold higher in metastatic MDA-MB-231 cells than in weakly metastatic MCF-7 cells. When voltage-gated membrane currents are examined in different cell lines, inward currents only occur in the highly metastatic breast cancer cell line MDA-MB-231 (Fraser et al., 2005). *In vivo*, it is widely acknowledged that Nav1.5 is present in breast cancer biopsies and is related to lymph node metastasis (Fraser et al., 2005). *SCN5A* expression is significantly elevated in breast cancer tissues and is an independent predictor of poor prognosis compared to its expression in normal breast tissue. *SCN5A* is overexpressed in tumor samples from patients who experience recurrence and death within 5 years; thus, *SCN5A* overexpression is associated with increased odds of developing metastasis (Yang et al., 2012). Nelson and his colleagues investigated the functional activity of Nav1.5 and its specific contribution to breast cancer tumor progression. *SCN5A* is upregulated at the mRNA and protein levels in metastatic breast tumors compared to that in normal, non-cancerous tissue (Nelson et al., 2015a; Nelson et al., 2015b; Yamaci et al., 2017)

Furthermore, some factors affect Nav1.5 expression in breast cancer cells. The β1 subunit mRNA and protein are strongly

expressed in MCF-7 cells and are barely detectable in MDA-MB-231 cells. Inhibition of the β1 subunit reduces adhesion and enhances metastatic cell behavior by upregulating nNav1.5 expression (Chioni et al., 2009). It has been reported that the expression level of repressor element silencing transcription factor (REST) is significantly lower in MDA-MB-231 cells than in MCF-7 cells (Kamarulzaman et al., 2017). The inhibition of REST results in re-expression of various neonatal genes (Kuwahara, 2013), and REST recruits histone deacetylases (HDACs) for transcriptional repression activity (Roopra et al., 2000). It has been postulated that downregulation of REST and HDAC2 expression levels enhances the expression of Nav1.5 and nNav1.5, promoting aggressiveness of tumors (Kamarulzaman et al., 2017). The sigma-1 receptor is known as a protein located on the plasma membrane, endoplasmic reticulum, and perinuclear areas (Hayashi and Su, 2003; Hayashi and Su, 2005), and is up-regulated in breast cancer cells and tissues (Aydar et al., 2016). In breast cancer cells, the sigma-1 receptor combines with nNav1.5 protein to form a complex with a four-fold symmetry (Balasuriya et al., 2012), which translocate nNav1.5 protein to the plasma membrane, thereby increases the metastatic activity (Aydar et al., 2016). Salt-inducible kinase 1 (SIK1) has been identified as an important factor in regulating sodium homeostasis and as a tumor repressor that participates in the progression of cancer cells (Sjostrom et al., 2007; Selvik et al., 2014). Lower expression of SIK1 was observed in different breast tumor grades than in normal tissues (Gradek et al., 2019). Silencing SIK1 upregulates *SCN5A* expression and increases H^+ outflow, thus, improving invasiveness (Gradek et al., 2019). TGF-β1 is a well-known inducer of epithelial-to-mesenchymal transition (EMT) and a multifunctional regulatory factor affecting cancer development (Jakowlew, 2006; Moustakas and Heldin, 2016). Weakly metastatic MCF-7 cells treated with TGF-β1 demonstrated increased expression of *SCN5A* and induction of invasion (Gradek et al., 2019). Epidermal growth factor (EGF) signals have been confirmed to be overexpressed and are involved in the development of breast cancer (Adams et al., 2009; Yao et al., 2012). Treatment with EGF promoted the migratory capacity of MDA-MB-231 cells by increasing the functional expression of the Nav1.5 channel (Gonzalez-Gonzalez et al., 2019). These results confirm that Nav1.5 channels play an important role in human breast cancer by affecting metastatic activity.

Furthermore, the importance of Nav1.5 expression in human breast cancer cells for the colonization of organs was assessed. ShCTL or shNav1.5 breast cancer cells injected into mice. ShCTL cells expressing Nav1.5 strongly colonized and invaded the chest area. All mice showed lung colonization and some showed rachis and rib colonization (Driffort et al., 2014). In addition, a high level of nNav1.5 expression is associated with the estrogen receptor (ERα) status of breast cancer. In all cases, lack of ERα was positive for nNav1.5 expression. In all cases of negative nNav1.5 expression, ERα is present (Yamaci et al., 2017). It is widely acknowledged that breast cancer cases lacking ERα protein are closely related to a more advanced stage and have a worse prognosis (Yi et al., 2014; Zhou and Slingerland, 2014). This is consistent with previous results showing that a high level of nNav1.5 expression is associated with aggressive breast cancer development. Consequently, nNav1.5 expression enhances growth and metastatic dissemination of breast cancer and is a potential prognostic marker for breast cancer.

Mechanisms of Action

Metastasis of breast cancer is a complex process, and there are a considerable number of studies focusing on this topic. Nav1.5 is involved in the metastatic cascade in breast cancer by acting on different targets.

Na^+ currents carried by Nav1.5 have been found to promote the invasiveness of the breast cancer cell line MDA-MB-231 by regulating the H^+ influx carried through Na^+/H^+ exchanger isoform-1 (NHE-1). NHE-1, which is an important regulator of H^+ efflux, is co-expressed with Nav1.5 in lipid rafts within the caveolae of MDA-MB-231 cells. NHE-1 and Nav1.5 are coupled in the metastatic process of MDA-MB-231 cells (Brisson et al., 2011). Na^+ inward currents can be found in highly metastatic MDA-MB-231 cells but are absent in weakly metastatic or normal cells. Similarly, highly metastatic MDA-MB-231 cells express a high level of NHE-1 mRNA, while weakly metastatic MCF-7 cells express a low level of NHE-1 mRNA, and normal cells express no NHE-1 (Chen et al., 2019). In MDA-MB-231 cells, Nav1.5 allosterically modulates the activity of NHE-1, rendering NHE-1 more active at physiological intracellular pH. This results in increased H^+ efflux to the extracellular space, leading to acidification of the peri-membrane and intracellular alkalization (Brisson et al., 2013; Amith and Fliegel, 2017). The environment of the perimembrane favors the proteolytic activity of cysteine cathepsins (Cat) and matrix metalloproteinases (MMPs), which are major drivers of extracellular matrix (ECM) degradation and increase the invasiveness of breast cancer cells (**Figure 1**). Cat C, Cat B, Cat K, and Cat L are involved in Cat-dependent invasiveness in MDA-MB-231 cells. Apart from Cat K, the activity of this proteolytic protein can be potentiated by Nav1.5 (Gillet et al., 2009; Robey and Nesbit, 2013). NHE-1–modulated invasiveness can also be found in hepatoma cells (Yang et al., 2011).

Nav1.5 expression enhances src kinase activity and controls cortactin phosphorylation, resulting in the release of actin-severing cofilin and the formation of actin barbed ends of invadopodias, which are actin-rich organelles that enable cells to stretch into the ECM and perform their proteolytic effect (Oser et al., 2009; Brisson et al., 2013). It is known that Nav1.5 expression in breast cancer cells is closely associated with the protein level of CD44, which correlates with poor outcomes of breast cancer (McFarlane et al., 2015; Nelson et al., 2015b). In turn, CD44 adheres to its ligand and leads to the activation of src and phosphorylation of cortactin (Bourguignon et al., 2001; Hill et al., 2006), thus, supporting that Nav1.5 may potentiate the invasion of breast cancer cells by the CD44-src-cortactin signaling axis. Apart from the CD44-src-cortactin signaling axis, CD44 targets NHE-1 to regulate the metastatic capacities of MDA-MB-231 cells through the mitogen-activated protein kinase pathway. Overexpression of CD44 upregulates the

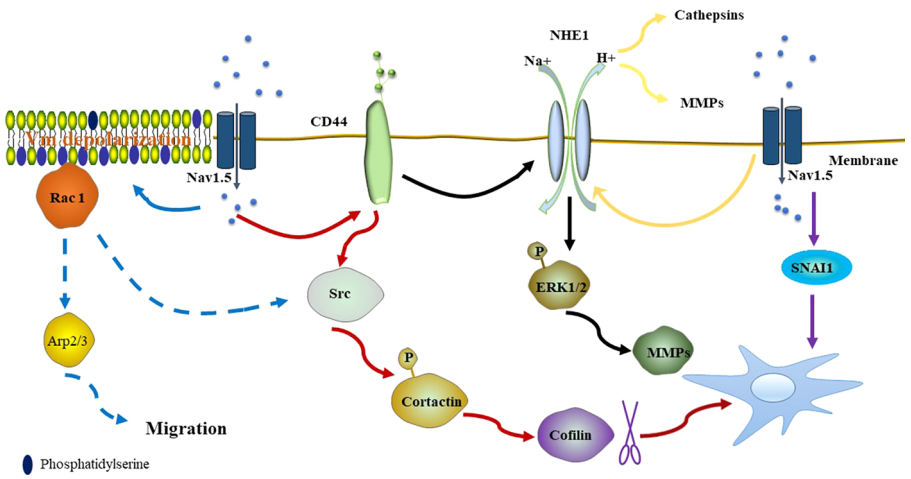


FIGURE 1 | Nav1.5 promotes the activity of NHE-1, resulting in the acidification of the peri-membrane, which favors the proteolytic activity of Cat and MMPs **(yellow line)**. Nav1.5 also promotes invasiveness by the CD44-src-cortactin signaling pathway. After phosphorylation, cortactin releases actin-severing cofilin, promoting the formation of actin barbed ends of invadopodia **(red line)**. Overexpression of CD44 upregulates the expression and activity of NHE-1, resulting in the expression level of MMPs (MMP2, MMP9, and MMP14) by increasing phosphorylated ERK1/2 **(black line)**. Nav1.5 dependent Vm depolarization regulates Rac1 activation by its interaction with phosphatidylserine **(blue solid line)**. Rac1 regulates the Arp2/3 complex and is closely associated with phosphorylation of cortactin and cofilin, promoting the promigratory phenotype **(blue dotted line)**. High expression of $Na_V1.5$ induces expression of SNAI1, triggering the EMT and pro-metastatic effect of breast cancer cells **(purple line)**. (Dotted line indicates that the mechanism exists but has not been confirmed in breast cancer).

expression and activity of NHE-1, resulting in the increased expression of MMPs (MMP2, MMP9, and MMP14) by increasing phosphorylated ERK1/2 (**Figure 1**). Moreover, CD44 accelerates tumor growth and metastasis to the lung in MDA-MB-231 cells (Chang et al., 2014).

Increasing evidence shows that plasma membrane potential (Vm) is related to cell cycle progression and the level of differentiation, development, regeneration, and migration of cancer cells (Sundelacruz et al., 2009; Yang and Brackenbury, 2013). Emerging evidence confirms that proliferating tumor cells depolarizes Vm, while terminally differentiated non-cancer cells are characterized by hyperpolarized Vm (Binggeli and Cameron, 1980; Marino et al., 1994; Fraser et al., 2005). Yang et al. reported that a steady-state inward Na^+ current carried by Nav1.5 contributes to depolarizing the resting Vm in MDA-MB-231 cells (Yang et al., 2020). Previous studies suggest that Vm depolarization promotes the redistribution of anionic phospholipids PIP_2 and phosphatidylserine within the plasma membrane, resulting in GTPase K-Ras activation (Chen et al., 2011; Zhou et al., 2015; Remorino et al., 2017). Rac1 is one of the Rho GTPases that contribute to lamellipodia formation and migration (Nobes and Hall, 1995; Steffen et al., 2013). Similarly, Nav1.5 dependent Vm depolarization regulates Rac1 activation and localization in lamellipodia by its interaction with phosphatidylserine, regulating cell morphology and migration in breast cancer cells (Yang et al., 2020). Rac1 is a potential upstream regulator of the Arp2/3 complex, which is required for lamellipodia extension (Suraneni et al., 2012). Furthermore, Rac1 is closely associated with phosphorylation of cortactin and cofilin, promoting the acquisition of a promigratory phenotype (Head et al., 2003; Sumida and Yamada, 2015) (**Figure 1**).

Na^+ currents carried by Nav1.5 stimulate phosphorylation of protein kinase A (PKA), which increases the Nav1.5 mRNA level but does not affect the total protein level. This changes the distribution of Nav1.5 in MDA-MB-231 cells, with the level of Nav1.5 increased on the plasma membrane and reduced in cytoplasm. The externalization of Nav1.5 potentiates the invasion and migration of breast cancer cells. The increase of Nav1.5 on the membrane in turn increases the Na^+ currents, thus, forming a positive feedback (**Figure 2**) (Chioni et al., 2010). There is also positive feedback between Nav1.5 and GTPase RhoA, which is overexpressed in many cancers, including breast cancer. Silencing of RhoA decreases the expression of Nav1.5 mRNA, as well as Nav1.5-mediated Na^+ currents. In turn, silencing of Nav1.5 decreased the protein level of RhoA (Dulong et al., 2014), which may be a result of the reduction in calcium concentration (Rao et al., 2001). In addition, suppressing both RhoA and Nav1.5 significantly reduced the invasion of MDA-MB-231 cells (Dulong et al., 2014).

Recent research has shown that Nav1.5 promotes invasiveness by taking part in EMT, a process involved in tissue formation during embryogenesis and repair processes, and promoting cancer cell dissemination from the primary tumor (Banyard and Bielenberg, 2015). MDA-MB-231 cells present typical spindle-shaped mesenchymal morphology and multiple filopodia. Upon knocking down the expression of *SCN5A*, these cells show decreased the number of filopodia that are shorter in length. The expression level of SNAI1, an EMT-inducing transcription factor, significantly decreased in MDA-MB-231 cells treated with a specific small-hairpin RNA (shNav1.5 cells). In contrast, MCF-7 cells overexpressing Nav1.5 display an increased invasive capacity, as well as an increased expression level of SNAI1, which provides evidence for

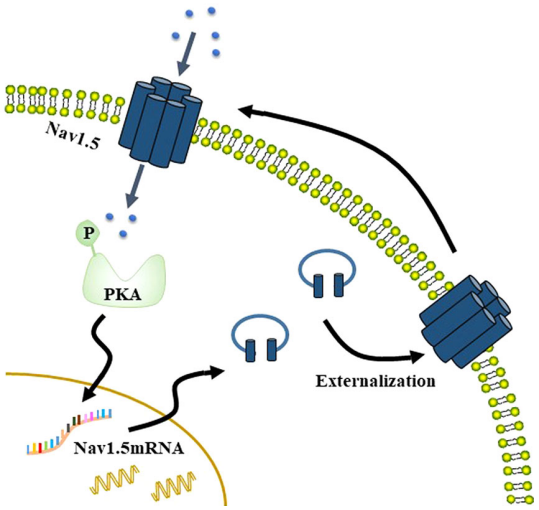


FIGURE 2 | Na^+ currents carried by Nav1.5 stimulate phosphorylation of PKA, which increases the Nav1.5 mRNA level but does not affect the total protein level. This changes the distribution of Nav1.5 in MDA-MB-231 cells, such that Nav1.5 protein level increases on the membrane but that in the cytoplasm decreases. The externalization of Nav1.5 then potentiates the invasion and migration of breast cancer cells. The increase of Nav1.5 on the membrane in turn increases the Na^+ currents, thus, forming a positive feedback.

the role of SNAI1 in Nav1.5 activity-mediated invasive process of breast cancer. Indeed, loss of expression of SIK1 upregulates Nav1.5 activity and expression (Gradek et al., 2019). Hence, it is that low expression levels of SIK1 in breast cancer cells induce the activity and expression of Nav1.5, followed by high expression of SNAI1, triggering the EMT and metastasis observed in breast cancer cells.

Inhibitors of Nav1.5

Nowadays, a variety of approaches, including specific antibodies, natural toxins, and pharmacological agents, are applied to reduce the metastasis of breast cancer cells by inhibiting Nav1.5 (**Table 1**).

Application of an anti-peptide polyclonal antibody, NESOpAb, blocks VGSC currents of EBNA-293 cells transfected with nNav1.5 and has no significant effect on adult Nav1.5 transfectants; thus, NESOpAb specifically targets nNav1.5, but not adult Nav1.5 (Chioni et al., 2005). NESOpAb inhibits the migration and invasion of MDA-MB-231 cells by suppressing the functional nNav1.5 activity but does not show similar results in human metastatic prostate cancer PC-3M cells, in which Nav1.7 is dominant (Diss et al., 2001; Brackenbury et al., 2007), which is consistent with the notion that NESOpAb specifically recognizes nNav1.5. E3Ab is a peptide-specific polyclonal antibody that recognizes the third extracellular region of Nav1.5, leading to specific inhibition of Nav1.5 activity (Xu et al., 2005). MDA-MB-231 cells treated with E3Ab show significantly decreased migration and invasive abilities. Indeed, a marked anti-proliferative effect was observed in E3Ab-treated MDA-MB-231 cells (Gao et al., 2019).

Phenytoin is an antiarrhythmic and antiepileptic agent that shows a high affinity toward Nav1.5 on the membrane of metastatic breast cancer cells in its inactivated state and reduces Na^+ currents. The depolarization of MDA-MB-231 causes the opening of VGSCs, which then rapidly enter an incomplete inactivated state after a few milliseconds. This process produces transient and persistent Na^+ currents, which play a key role in the metastasis of breast cancer cells (Yang et al., 2012). Phenytoin significantly reduces the mobility, migration, and invasion of metastatic breast cancer *in vitro* and *in vivo* (Nelson et al., 2015a; Mohammed et al., 2016). Blocking the Na^+ current by phenytoin results in the downregulation of EGF expression and EGF-induced ERK1/2 phosphorylation, reducing the activity of MMP7, cathepsin E, and kallikrein-10, among which phenytoin showed the greatest inhibitory effect on cathepsin E (Mohammed et al., 2016). It has been proven that higher cathepsin E levels in the serum are positively correlated with poorer clinical prognosis in breast cancer patients (Kawakubo et al., 2014), thus, phenytoin might serve as an

TABLE 1 | Inhibitors of Nav1.5.

Inhibitor	Type	Effects on cancer cells	Mechanism	References
NESOpb/ E3Ab	Antibodies	Reduce migration and invasion	Specifically inhibit $Na_V1.5$	Brackenbury et al. (2007) Gao et al. (2019)
Phenytoin	Antiarrhythmic and antiepileptic agent	Reduce mobility migration and invasion	Inhibit Na+ currents down-regulate EGF expression and ERK1/2 phosphorylation reduce activity of MP-7, cathepsin E and kallikrein-10	Yang et al. (2012) Mohammed et al. (2016) Nelson et al. (2015a)
DAPT	Notch signal inhibitor	Reduce proliferation lateral motility	Increase the level of Notch4mRNA and decreased the ratio of MMP9 to TIMP1	Aktas et al. (2015)
DHA	ω-3 LC-PUFA	Reduce migration and invasion	Inhibit the expression of PPARβ and downregulate SCN5A expression and activity of NHE-1	Isbilen et al. (2006) Wannous et al. (2015)
Ranolazine	Antiarrhythmic drug	Reduce invasion	Decreases Na+ influx and ECM degradative activity	Driffort et al. (2014)
Taxol	Anticancer drug	Decrease invasion	Left shift of $Na_V1.5$ activation and affect microtubule polymerization	Tran et al. (2009)
FS50	Animal salivary protein	Reduce migration and invasion	Decrease the expression of $Na_V1.5$ mRNA level and distribution of $Na_V1.5$ protein in the cell membrane	Zhang et al. (2018)
Compounds	Synthetic substances	Reduce the invasion	Block Nav1.5 channel	Dutta et al. (2018)
Propranolol	β-AR blocker	Reduce migration and invasion	Affect the properties and expression of Nav1.5	Lee et al. (2019)

effective drug for improving therapeutic efficiency and survival of patients (Mohammed et al., 2016). Although phenytoin does not affect proliferation *in vitro* (Yang et al., 2012), it reduces the proliferation of metastatic breast cancer and slows tumor growth *in vivo* (Nelson et al., 2015a). The Notch signal inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine-*t*-butyl ester (DAPT) has an anti-proliferative effect on MDA-MB-231 cells that can be decreased by phenytoin. Both DAPT and phenytoin reduce the lateral motility of breast cancer cells. It is reasonable to speculate that they have different effects on the proliferation of breast cancer cells. DAPT reduces the nNav1.5 mRNA expression level, which is not observed in the phenytoin treatment group. DAPT significantly reduces the ratio of MMP9 to tissue inhibitor of metalloproteinases-1 (TIMP1), which could be partly reversed by the combination of DAPT and phenytoin, indicating that the application of DAPT together with phenytoin is not a better choice than the single treatment of DAPT and phenytoin (Aktas et al., 2015). Furthermore, phenytoin inhibits the metastasis of breast cancer cells to other organs such as the lungs, liver, and spleen by reducing the density of MMP9-expressing cells (Nelson et al., 2015a). This suggests that the contribution of Nav1.5 to tumor growth *in vivo* is closely associated with adjacent tissues or the ECM.

It is noteworthy that some dietary habits have been shown to protect against cancer. It has been reported that omega-3 long-chain polyunsaturated fatty acids (ω-3 LC-PUFAs) play a role in the prevention of cancer. The effects of docosahexaenoic acid (DHA), one of the most important ω-3 LC-PUFAs, have been studied in the field of breast cancer. Short- and long-term applications of DHA inhibit nNav1.5 activity in the metastatic human MDA-MB-231 breast cancer cell line by decreasing the peak VGSC current density. In the long-term application, DHA inhibits both mRNA and protein expression levels of nNav1.5 and reduces cell migration (Isbilen et al., 2006). Peroxisome proliferator-activated receptor β (PPARβ) regulates the expression of numerous genes, including *SCN5A*, by binding with PPAR response elements on the promoters of these genes, thus, regulating cell survival, inflammation, and metabolism (Peters et al., 2012). DHA, a natural ligand of PPAR, has shown the potential to suppress tumor growth in different kinds of models with no side effects (Forman et al., 1997; Yao et al., 2014). DHA has been shown to reduce PPARβ expression, which is overexpressed in breast cancer cells, and inhibits the invasiveness and growth of breast cancer cells (Wannous et al., 2013; Yao et al., 2014; Wannous et al., 2015). The downregulation of PPARβ reduces *SCN5A* expression at both the mRNA and protein levels, as well as nNav1.5 density at the plasma membrane, which finally leads to the reduction of Na^+ currents. In addition, the downregulation of PPARβ reduces NHE-1–dependent H^+ efflux by inhibiting the activity of Nav1.5 channels without altering NHE-1 expression. PPARβ is indispensable for DHA to reduce Nav1.5 expression and NHE-1 activity, giving rise to the invasiveness of breast cancer cells (Wannous et al., 2015).

Ranolazine has been approved by the US Food and Drug Administration for chronic angina (Antzelevitch et al., 2004). One of the most characterized pharmacological effects of ranolazine is its ability to selectively inhibit late Na^+ currents. The inhibition of Na^+ currents triggers a steeper Na^+ gradient and activation of the Na^+/Ca exchanger, which reduces the intracellular overloaded calcium and improves ventricular relaxation in cardiac ischemia conditions (Fraser et al., 2006). Ranolazine has also been shown to have anti-invasiveness potential in breast cancer. *In vitro*, ranolazine reduces the function of Nav1.5 and decreases Na^+ influx in MDA-MB-231 cells, which results in decreased ECM degradative activity and pro-invasive morphology of cells. *In vivo*, ranolazine slows down tumor growth and inhibits the colonization of breast cancer cells to other organs without obvious toxic effects by reducing the Nav1.5 carried currents in tumor tissues (Driffort et al., 2014). The expressions of β-adrenergic receptor (β-AR) and Nav1.5 overlap substantially in MDA-MB-231 cells. Propranolol which is a blocker of β-AR, coupled to with PKA activation, modulates Nav1.5 and takes part in reducing migration and invasion of MDA-MB-231 cells. Short-term treatment with propranolol tends to reduce peak Na^+ currents carried by Nav1.5, while long-term treatment results in sustained changes of properties and expression of Nav1.5. Indeed, the role of factors contained in the serum cannot be ignored. Both propranolol and ranolazine reduced the motility and invasiveness of MDA-MB-231 cells, but the effect of their combination was not better than that of individual treatments (Lee et al., 2019).

Taxol and its derivatives, such as docetaxel, are widely used in the treatment of breast cancer (Dang and Hudis, 2006). The widely accepted explanation for their effects is that they stabilize the microtubules during mitosis, which leads to the inhibition of cell division (Camacho et al., 2000). Taxol also has a potential anti-invasive effect on breast cancer cells at low concentrations but has no effect on cell proliferation. The reduction of the current carried by Nav1.5 was not observed in cells pretreated with taxol, which demonstrates a significant leftward shift of the activation properties of Nav1.5. It is known that the activity of sodium channels is closely associated with perturbation of microtubules (Motlagh et al., 2002). Tran reported that a short-term and low-dose taxol treatment is sufficient to affect microtubule polymerization (Tran et al., 2009). It seems that the effects of taxol on cell invasiveness are complicated, and other proteins involved in the Nav1.5-regulated signaling pathway may be regulated by taxol, but the precise mechanisms remain unknown.

Recently, animal peptides have drawn considerable interest. FS50, the salivary protein from *Xenopsylla cheopis*, shows an inhibitory effect against the Nav1.5 channel (Xu et al., 2016). FS50 significantly reduced the peak VGSC currents and decreased the expression of Nav1.5 mRNA in MDA-MB-231 cells but had no effect on the total Nav1.5 protein levels. Notably, FS50 reduces the Nav1.5 protein expression in the plasma membrane, which means that FS50 only changes the distribution of the protein between the plasma membrane and the cytoplasm. The effect is identical to the effects of a PKA inhibitor; thus, FS50 may inhibit the PKA pathway, resulting in the reduced expression of Nav1.5. In the future, it is worth

detecting PKA-related proteins after the treatment of FS50 (Brackenbury and Djamgoz, 2006; Chioni et al., 2010). FS50 inhibits the migration and invasion of MDA-MB-231 cells but has no effect on the proliferation of MCF-7 and MDA-MB-231 cells. The reduction of MMP9 activity and the ratio of MMP9 mRNA to TIMP1 mRNA were observed in MDA-MB-231 cells treated with FS50 (Zhang et al., 2018).

In addition to the known VGSC-blocking drugs, scientists have developed a highly predictive and comprehensive three-dimensional quantitative structure–activity relationship model for designing the compounds to bind with VGSC ligands (Zha et al., 2014). Five low micromolar, small molecule compounds acting as Nav1.5 blockers in MDA-MB-231 cells were designed, synthesized, and evaluated. Two of the compounds were identified to reduce peak Na^+ currents and reduce the invasion of MDA-MB-231 cells without affecting cell viability (Dutta et al., 2018). Compared with known drugs, these compounds are more effective and have simpler chemical structures. Moreover, synthetic substances are designed to target Nav1.5, providing a new and potent direction to develop drugs for the treatment of breast cancer metastasis.

The membrane current generated by VGSCs has two distinct modes: transient (I_{NaT}) and persistent (I_{NaP}). I_{NaP} can last 100 ms to 1 s, while I_{NaT} lasts a millisecond (Djamgoz and Onkal, 2013). Hypoxia, which is commonly observed in cancer and promotes metastasis and invasion, significantly contributes to increasing I_{NaP} (Hammarstrom and Gage, 2002; Muz et al., 2015; Rankin and Giaccia, 2016; Guzel et al., 2019). Compared with I_{NaT}, I_{NaP} is resistant to inactivation even at depolarized potentials and will lead to significant changes in the global level of Na^+ that affect Nav1.5-dependent mechanisms and play an important role in cancer progression (Saint, 2006). Indeed, the inhibition of I_{NaP} has been shown to produce a major anti-metastatic effect and has been proposed as a new target (Guzel et al., 2019). As mentioned above, MDA-MB-231 cells exhibit depolarized Vm. In addition, slower kinetics of activation, inactivation, and recovery from inactivation are observed in nNav1.5 than in the 'adult' form (Onkal et al., 2008). Thus, it is reasonable to assume that I_{NaP} is mainly responsible for Na^+ elevation in breast cancer cells. At present, it is easy to identify I_{NaT} and I_{NaP} by whole-cell patch-clamp recordings, and a number of pharmacological agents have been shown to selectively block I_{NaP} (Urbani and Belluzzi, 2000; Maier, 2009). In the future, additional I_{NaP} blockers inhibiting Nav1.5-mediated persistent current should be considered as a new target to reduce metastasis of breast cancer. In addition, most agents listed above produce a major anti-metastatic effect by decreasing the current carried by Nav1.5 or changing the properties of Nav1.5. Specifically, decreasing the expression of nNav1.5 is another ideal target for specific anti-breast cancer treatment.

PERSPECTIVES

The increasing evidence is indicative of the role of aberrant Nav1.5 activation in the metastatic progression of breast cancer cells. Nav1.5 functions to trigger a variety of downstream mechanisms in breast cancer cells to regulate metastatic and invasive capacity. Considering the large and growing body of evidence, different approaches recognizing Nav1.5 are applied to inhibit metastasis of breast cancer *in vitro* and favor Nav1.5 as an anti-metastatic target. Although we reviewed several studies here, there are many unanswered questions that require further investigation: (i) Is it possible to make nNav1.5 a biomarker of early diagnosis of breast cancer; (ii) Can we develop additional agents specifically blocking I_{NaP} or nNav1.5 to improve efficacy and reduce side effects; and (iii) How can we accumulate the process of translational medicine and promote the application of these agents in the clinic to improve the outcomes for breast cancer patients.

AUTHOR CONTRIBUTIONS

QL and TW wrote the review and designed the figures and the table. WW and GC made contributions to searching related articles. XL, LJ, HT, MR and SK contributed to the conception of the study. MD supervised the whole work, contributed to writing, and critically revised the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China under contract (Nos. 31672290, 31100764, 31971190, 30901874, 81503276), the Natural Science Foundation of Hunan Province, China (No. 2016JJ3180), the Valuable Instrument and Equipment Fund of Central South University (No. CSUZC2019046, CSUZC2020043).

REFERENCES

Adams, B. D., Cowee, D. M., and White, B. A. (2009). The role of miR-206 in the epidermal growth factor (EGF) induced repression of estrogen receptor-alpha (ERalpha) signaling and a luminal phenotype in MCF-7 breast cancer cells. *Mol. Endocrinol.* 23, 1215–1230. doi: 10.1210/me.2009-0062

Aktas, C. C., Zeybek, N. D., and Piskin, A. K. (2015). In vitro effects of phenytoin and DAPT on MDA-MB-231 breast cancer cells. *Acta Biochim. Biophys. Sin. (Shanghai)* 47, 680–686. doi: 10.1093/abbs/gmv066

Amin, A. S., Pinto, Y. M., and Wilde, A. A. (2013). Long QT syndrome: beyond the causal mutation. *J. Physiol.* 591, 4125–4139. doi: 10.1113/jphysiol.2013.254920

Amith, S. R., and Fliegel, L. (2017). Na(+)/H(+) exchanger-mediated hydrogen ion extrusion as a carcinogenic signal in triple-negative breast cancer

etiopathogenesis and prospects for its inhibition in therapeutics. *Semin. Cancer Biol.* 43, 35–41. doi: 10.1016/j.semcancer.2017.01.004

Antzelevitch, C., Belardinelli, L., Zygmunt, A. C., Burashnikov, A., Di Diego, J. M., Fish, J. M., et al. (2004). Electrophysiological effects of ranolazine, a novel antianginal agent with antiarrhythmic properties. *Circulation* 110, 904–910. doi: 10.1161/01.CIR.0000139333.83620.5D

Antzelevitch, C., Brugada, P., Borggrefe, M., Brugada, J., Brugada, R., Corrado, D., et al. (2005). Brugada syndrome: report of the second consensus conference. *Heart Rhythm.* 2, 429–440. doi: 10.1016/j.hrthm.2005.01.005

Arciero, C. A., Guo, Y., Jiang, R., Behera, M., O'Regan, R., Peng, L., et al. (2019). ER(+)/HER2(+) Breast Cancer Has Different Metastatic Patterns and Better Survival Than ER(-)/HER2(+) Breast Cancer. *Clin. Breast Cancer* 19, 236–245. doi: 10.1016/j.clbc.2019.02.001

Aydar, E., Stratton, D., Fraser, S. P., Djamgoz, M. B., and Palmer, C. (2016). Sigma-1 receptors modulate neonatal Nav1.5 ion channels in breast cancer cell lines. *Eur. Biophys. J.* 45, 671–683. doi: 10.1007/s00249-016-1135-0

Balasuriya, D., Stewart, A. P., Crottes, D., Borgese, F., Soriani, O., and Edwardson, J. M. (2012). The sigma-1 receptor binds to the Nav1.5 voltage-gated Na+ channel with 4-fold symmetry. *J. Biol. Chem.* 287, 37021–37029. doi: 10.1074/jbc.M112.382077

Banyard, J., and Bielenberg, D. R. (2015). The role of EMT and MET in cancer dissemination. *Connect Tissue Res.* 56, 403–413. doi: 10.3109/03008207.2015.1060970

Beckermann, T. M., McLeod, K., Murday, V., Potet, F., and George, A. L.Jr. (2014). Novel SCN5A mutation in amiodarone-responsive multifocal ventricular ectopy-associated cardiomyopathy. *Heart Rhythm.* 11, 1446–1453. doi: 10.1016/j.hrthm.2014.04.042

Binggeli, R., and Cameron, I. L. (1980). Cellular potentials of normal and cancerous fibroblasts and hepatocytes. *Cancer Res.* 40, 1830–1835.

Black, J. A., and Waxman, S. G. (2013). Noncanonical roles of voltage-gated sodium channels. *Neuron* 80, 280–291. doi: 10.1016/j.neuron.2013.09.012

Bourguignon, L. Y., Zhu, H., Shao, L., and Chen, Y. W. (2001). CD44 interaction with c-Src kinase promotes cortactin-mediated cytoskeleton function and hyaluronic acid-dependent ovarian tumor cell migration. *J. Biol. Chem.* 276, 7327–7336. doi: 10.1074/jbc.M006498200

Brackenbury, W. J., and Djamgoz, M. B. (2006). Activity-dependent regulation of voltage-gated Na+ channel expression in Mat-LyLu rat prostate cancer cell line. *J. Physiol.* 573, 343–356. doi: 10.1113/jphysiol.2006.106906

Brackenbury, W. J., and Isom, L. L. (2011). Na Channel beta Subunits: Overachievers of the Ion Channel Family. *Front. Pharmacol.* 2, 53. doi: 10.3389/fphar.2011.00053

Brackenbury, W. J., Chioni, A. M., Diss, J. K., and Djamgoz, M. B. (2007). The neonatal splice variant of Nav1.5 potentiates in vitro invasive behaviour of MDA-MB-231 human breast cancer cells. *Breast Cancer Res. Treat* 101, 149–160. doi: 10.1007/s10549-006-9281-1

Breastcancer.org. *Breast Cancer Statistics*. Available at: https://www.breastcancer.org/symptoms/understand_bc/statistics

Brisson, L., Gillet, L., Calaghan, S., Besson, P., Le Guennec, J. Y., Roger, S., et al. (2011). Na(V)1.5 enhances breast cancer cell invasiveness by increasing NHE1-dependent H(+) efflux in caveolae. *Oncogene* 30, 2070–2076. doi: 10.1038/onc.2010.574

Brisson, L., Driffort, V., Benoist, L., Poet, M., Counillon, L., Antelmi, E., et al. (2013). NaV1.5 Na(+) channels allosterically regulate the NHE-1 exchanger and promote the activity of breast cancer cell invadopodia. *J. Cell Sci.* 126, 4835–4842. doi: 10.1242/jcs.123901

Camacho, J., Sanchez, A., Stuhmer, W., and Pardo, L. A. (2000). Cytoskeletal interactions determine the electrophysiological properties of human EAG potassium channels. *Pflugers Arch.* 441, 167–174. doi: 10.1007/s004240000420

Catterall, W. A. (2000). From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. *Neuron* 26, 13–25. doi: 10.1016/S0896-6273(00)81133-2

Chang, G., Wang, J., Zhang, H., Zhang, Y., Wang, C., Xu, H., et al. (2014). CD44 targets Na(+)/H(+) exchanger 1 to mediate MDA-MB-231 cells' metastasis via the regulation of ERK1/2. *Br. J. Cancer* 110, 916–927. doi: 10.1038/bjc.2013.809

Chen, X., Zhang, X., Jia, C., Xu, J., Gao, H., Zhang, G., et al. (2011). Membrane depolarization increases membrane PtdIns(4,5)P2 levels through mechanisms involving PKC betaII and PI4 kinase. *J. Biol. Chem.* 286, 39760–39767. doi: 10.1074/jbc.M111.289090

Chen, Q., Liu, Y., Zhu, X. L., Feng, F., Yang, H., and Xu, W. (2019). Increased NHE1 expression is targeted by specific inhibitor cariporide to sensitize resistant breast cancer cells to doxorubicin in vitro and in vivo. *BMC Cancer* 19, 211. doi: 10.1186/s12885-019-5397-7

Chioni, A. M., Fraser, S. P., Pani, F., Foran, P., Wilkin, G. P., Diss, J. K., et al. (2005). A novel polyclonal antibody specific for the Na(v)1.5 voltage-gated Na (+) channel 'neonatal' splice form. *J. Neurosci. Methods* 147, 88–98. doi: 10.1016/j.jneumeth.2005.03.010

Chioni, A. M., Brackenbury, W. J., Calhoun, J. D., Isom, L. L., and Djamgoz, M. B. (2009). A novel adhesion molecule in human breast cancer cells: voltage-gated Na+ channel beta1 subunit. *Int. J. Biochem. Cell Biol.* 41, 1216–1227. doi: 10.1016/j.biocel.2008.11.001

Chioni, A. M., Shao, D., Grose, R., and Djamgoz, M. B. (2010). Protein kinase A and regulation of neonatal Nav1.5 expression in human breast cancer cells: activity-dependent positive feedback and cellular migration. *Int. J. Biochem. Cell Biol.* 42, 346–358. doi: 10.1016/j.biocel.2009.11.021

Dang, C., and Hudis, C. (2006). Adjuvant taxanes in the treatment of breast cancer: no longer at the tip of the iceberg. *Clin. Breast Cancer* 7, 51–58. doi: 10.3816/CBC.2006.n.013

Diss, J. K., Archer, S. N., Hirano, J., Fraser, S. P., and Djamgoz, M. B. (2001). Expression profiles of voltage-gated Na(+) channel alpha-subunit genes in rat and human prostate cancer cell lines. *Prostate* 48, 165–178. doi: 10.1002/pros.1095

Djamgoz, M. B., and Onkal, R. (2013). Persistent current blockers of voltage-gated sodium channels: a clinical opportunity for controlling metastatic disease. *Recent Pat. Anticancer Drug Discovery* 8, 66–84. doi: 10.2174/1574892811308010066

Driffort, V., Gillet, L., Bon, E., Marionneau-Lambot, S., Oullier, T., Joulin, V., et al. (2014). Ranolazine inhibits NaV1.5-mediated breast cancer cell invasiveness and lung colonization. *Mol. Cancer* 13, 264. doi: 10.1186/1476-4598-13-264

Dulong, C., Fang, Y. J., Gest, C., Zhou, M. H., Patte-Mensah, C., Mensah-Nyagan, A. G., et al. (2014). The small GTPase RhoA regulates the expression and function of the sodium channel Nav1.5 in breast cancer cells. *Int. J. Oncol.* 44, 539–547. doi: 10.3892/ijo.2013.2214

Dutta, S., Lopez Charcas, O., Tanner, S., Gradek, F., Driffort, V., Roger, S., et al. (2018). Discovery and evaluation of nNav1.5 sodium channel blockers with potent cell invasion inhibitory activity in breast cancer cells. *Bioorg. Med. Chem.* 26, 2428–2436. doi: 10.1016/j.bmc.2018.04.003

Fairhurst, C., Watt, I., Martin, F., Bland, M., and Brackenbury, W. J. (2015). Sodium channel-inhibiting drugs and survival of breast, colon and prostate cancer: a population-based study. *Sci. Rep.* 5, 16758. doi: 10.1038/srep16758

Forman, B. M., Chen, J., and Evans, R. M. (1997). Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. *Proc. Natl. Acad. Sci. U.S.A.* 94, 4312–4317. doi: 10.1073/pnas.94.9.4312

Fraser, S. P., Diss, J. K., Chioni, A. M., Mycielska, M. E., Pan, H., Yamaci, R. F., et al. (2005). Voltage-gated sodium channel expression and potentiation of human breast cancer metastasis. *Clin. Cancer Res.* 11, 5381–5389. doi: 10.1158/1078-0432.CCR-05-0327

Fraser, H., Belardinelli, L., Wang, L., Light, P. E., McVeigh, J. J., and Clanachan, A. S. (2006). Ranolazine decreases diastolic calcium accumulation caused by ATX-II or ischemia in rat hearts. *J. Mol. Cell Cardiol.* 41, 1031–1038. doi: 10.1016/j.yjmcc.2006.08.012

Gao, R., Cao, T., Chen, H., Cai, J., Lei, M., and Wang, Z. (2019). Nav1.5-E3 antibody inhibits cancer progression. *Trans. Cancer Res.* 8, 44–50. doi: 10.21037/tcr.2018.12.23

Gillet, L., Roger, S., Besson, P., Lecaille, F., Gore, J., Bougnoux, P., et al. (2009). Voltage-gated Sodium Channel Activity Promotes Cysteine Cathepsin-dependent Invasiveness and Colony Growth of Human Cancer Cells. *J. Biol. Chem.* 284, 8680–8691. doi: 10.1074/jbc.M806891200

Giudicessi, J. R., and Ackerman, M. J. (2013). Genotype- and phenotype-guided management of congenital long QT syndrome. *Curr. Probl. Cardiol.* 38, 417–455. doi: 10.1016/j.cpcardiol.2013.08.001

Gonzalez-Gonzalez, L., Gonzalez-Ramirez, R., Flores, A., Avelino-Cruz, J. E., Felix, R., and Monjaraz, E. (2019). Epidermal Growth Factor Potentiates Migration of MDA-MB 231 Breast Cancer Cells by Increasing NaV1.5 Channel Expression. *Oncology* 97, 373–382. doi: 10.1159/000501802

Gradek, F., Lopez-Charcas, O., Chadet, S., Poisson, L., Ouldamer, L., Goupille, C., et al. (2019). Sodium Channel Nav1.5 Controls Epithelial-to-Mesenchymal

Transition and Invasiveness in Breast Cancer Cells Through its Regulation by the Salt-Inducible Kinase-1. *Sci. Rep.* 9, 18652. doi: 10.1038/s41598-019-55197-5

Greaney, M. L., Sprunck-Harrild, K., Ruddy, K. J., Ligibel, J., Barry, W. T., Baker, E., et al. (2015). Study protocol for Young & Strong: a cluster randomized design to increase attention to unique issues faced by young women with newly diagnosed breast cancer. *BMC Public Health* 15, 37. doi: 10.1186/s12889-015-1346-9

Guzel, R. M., Ogmen, K., Ilieva, K. M., Fraser, S. P., and Djamgoz, M. B. A. (2019). Colorectal cancer invasiveness in vitro: Predominant contribution of neonatal Nav1.5 under normoxia and hypoxia. *J. Cell Physiol.* 234, 6582–6593. doi: 10.1002/jcp.27399

Hammarstrom, A. K., and Gage, P. W. (2002). Hypoxia and persistent sodium current. *Eur. Biophys. J.* 31, 323–330. doi: 10.1007/s00249-002-0218-2

Hayashi, T., and Su, T. P. (2003). Sigma-1 receptors (sigma(1) binding sites) form raft-like microdomains and target lipid droplets on the endoplasmic reticulum: roles in endoplasmic reticulum lipid compartmentalization and export. *J. Pharmacol. Exp. Ther.* 306, 718–725. doi: 10.1124/jpet.103.051284

Hayashi, T., and Su, T. (2005). The sigma receptor: evolution of the concept in neuropsychopharmacology. *Curr. Neuropharmacol.* 3, 267–280. doi: 10.2174/157015905774322516

Head, J. A., Jiang, D., Li, M., Zorn, L. J., Schaefer, E. M., Parsons, J. T., et al. (2003). Cortactin tyrosine phosphorylation requires Rac1 activity and association with the cortical actin cytoskeleton. *Mol. Biol. Cell* 14, 3216–3229. doi: 10.1091/mbc.e02-11-0753

Hill, A., McFarlane, S., Mulligan, K., Gillespie, H., Draffin, J. E., Trimble, A., et al. (2006). Cortactin underpins CD44-promoted invasion and adhesion of breast cancer cells to bone marrow endothelial cells. *Oncogene* 25, 6079–6091. doi: 10.1038/sj.onc.1209628

Hoshino, A., Costa-Silva, B., Shen, T. L., Rodrigues, G., Hashimoto, A., Tesic Mark, M., et al. (2015). Tumour exosome integrins determine organotropic metastasis. *Nature* 527, 329–335. doi: 10.1038/nature15756

Hull, J. M., and Isom, L. L. (2018). Voltage-gated sodium channel beta subunits: The power outside the pore in brain development and disease. *Neuropharmacology* 132, 43–57. doi: 10.1016/j.neuropharm.2017.09.018

Hurvitz, S., Singh, R., Adams, B., Taguchi, J. A., Chan, D., Dichmann, R. A., et al. (2018). Phase Ib/II single-arm trial evaluating the combination of everolimus, lapatinib and capecitabine for the treatment of HER2-positive breast cancer with brain metastases (TRIO-US B-09). *Ther. Adv. Med. Oncol.* 10, 1–13. doi: 10.1177/1758835918807339

Isbilen, B., Fraser, S. P., and Djamgoz, M. B. (2006). Docosahexaenoic acid (omega-3) blocks voltage-gated sodium channel activity and migration of MDA-MB-231 human breast cancer cells. *Int. J. Biochem. Cell Biol.* 38, 2173–2182. doi: 10.1016/j.biocel.2006.06.014

Jakowlew, S. B. (2006). Transforming growth factor-beta in cancer and metastasis. *Cancer Metastasis Rev.* 25, 435–457. doi: 10.1007/s10555-006-9006-2

Kamarulzaman, N. S., Dewadas, H. D., Leow, C. Y., Yaacob, N. S., and Mokhtar, N. F. (2017). The role of REST and HDAC2 in epigenetic dysregulation of Nav1.5 and nNav1.5 expression in breast cancer. *Cancer Cell Int.* 17, 74. doi: 10.1186/s12935-017-0442-6

Kao, L. T., Huang, C. C., Lin, H. C., and Huang, C. Y. (2018). Antiarrhythmic drug usage and prostate cancer: a population-based cohort study. *Asian J. Androl.* 20, 37–42. doi: 10.4103/aja.aja_26_17

Kawakubo, T., Yasukochi, A., Toyama, T., Takahashi, S., Okamoto, K., Tsukuba, T., et al. (2014). Repression of cathepsin E expression increases the risk of mammary carcinogenesis and links to poor prognosis in breast cancer. *Carcinogenesis* 35, 714–726. doi: 10.1093/carcin/bgt373

Kuwahara, K. (2013). Role of NRSF/REST in the regulation of cardiac gene expression and function. *Circ. J.* 77, 2682–2686. doi: 10.1253/circj.CJ-13-1210

Lee, A., Fraser, S. P., and Djamgoz, M. B. A. (2019). Propranolol inhibits neonatal Nav1.5 activity and invasiveness of MDA-MB-231 breast cancer cells: Effects of combination with ranolazine. *J. Cell Physiol.* 234, 23066–23081. doi: 10.1002/jcp.28868

Maier, L. S. (2009). A novel mechanism for the treatment of angina, arrhythmias, and diastolic dysfunction: inhibition of late I(Na) using ranolazine. *J. Cardiovasc. Pharmacol.* 54, 279–286. doi: 10.1097/FJC.0b013e3181a1b9e7

Margan, M. M., Jitariu, A. A., Cimpean, A. M., Nica, C., and Raica, M. (2016). Molecular Portrait of the Normal Human Breast Tissue and Its Influence on Breast Carcinogenesis. *J. Breast Cancer* 19, 99–111. doi: 10.4048/jbc.2016.19.2.99

Marino, A. A., Morris, D. M., Schwalke, M. A., Iliev, I. G., and Rogers, S. (1994). Electrical potential measurements in human breast cancer and benign lesions. *Tumour Biol.* 15, 147–152. doi: 10.1159/000217885

Massague, J., Batlle, E., and Gomis, R. R. (2017). Understanding the molecular mechanisms driving metastasis. *Mol. Oncol.* 11, 3–4. doi: 10.1002/1878-0261.12024

McFarlane, S., Coulter, J. A., Tibbits, P., O'Grady, A., McFarlane, C., Montgomery, N., et al. (2015). CD44 increases the efficiency of distant metastasis of breast cancer. *Oncotarget* 6, 11465–11476. doi: 10.18632/oncotarget.3410

Mego, M., Mani, S. A., and Cristofanilli, M. (2010). Molecular mechanisms of metastasis in breast cancer–clinical applications. *Nat. Rev. Clin. Oncol.* 7, 693–701. doi: 10.1038/nrclinonc.2010.171

Mohammed, F. H., Khajah, M. A., Yang, M., Brackenbury, W. J., and Luqmani, Y. A. (2016). Blockade of voltage-gated sodium channels inhibits invasion of endocrine-resistant breast cancer cells. *Int. J. Oncol.* 48, 73–83. doi: 10.3892/ijo.2015.3239

Monk, M., and Holding, C. (2001). Human embryonic genes re-expressed in cancer cells. *Oncogene* 20, 8085–8091. doi: 10.1038/sj.onc.1205088

Motlagh, D., Alden, K. J., Russell, B., and Garcia, J. (2002). Sodium current modulation by a tubulin/GTP coupled process in rat neonatal cardiac myocytes. *J. Physiol.* 540, 93–103. doi: 10.1113/jphysiol.2001.013474

Moustakas, A., and Heldin, C. H. (2016). Mechanisms of TGFbeta-Induced Epithelial-Mesenchymal Transition. *J. Clin. Med.* 5, 63. doi: 10.3390/jcm5070063

Muz, B., de la Puente, P., Azab, F., and Azab, A. K. (2015). The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. *Hypoxia (Auckl)* 3, 83–92. doi: 10.2147/HP.S93413

Nelson, M., Yang, M., Dowle, A. A., Thomas, J. R., and Brackenbury, W. J. (2015a). The sodium channel-blocking antiepileptic drug phenytoin inhibits breast tumour growth and metastasis. *Mol. Cancer* 14, 13. doi: 10.1186/s12943-014-0277-x

Nelson, M., Yang, M., Millican-Slater, R., and Brackenbury, W. J. (2015b). Nav1.5 regulates breast tumor growth and metastatic dissemination in vivo. *Oncotarget* 6, 32914–32929. doi: 10.18632/oncotarget.5441

Nobes, C. D., and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 81, 53–62. doi: 10.1016/0092-8674(95)90370-4

Onkal, R., Mattis, J. H., Fraser, S. P., Diss, J. K., Shao, D., Okuse, K., et al. (2008). Alternative splicing of Nav1.5: an electrophysiological comparison of 'neonatal' and 'adult' isoforms and critical involvement of a lysine residue. *J. Cell Physiol.* 216, 716–726. doi: 10.1002/jcp.21451

Oser, M., Yamaguchi, H., Mader, C. C., Bravo-Cordero, J. J., Arias, M., Chen, X., et al. (2009). Cortactin regulates cofilin and N-WASp activities to control the stages of invadopodium assembly and maturation. *J. Cell Biol.* 186, 571–587. doi: 10.1083/jcb.200812176

Park, S. B., Chung, C. K., Gonzalez, E., and Yoo, C. (2018). Causal Inference Network of Genes Related with Bone Metastasis of Breast Cancer and Osteoblasts Using Causal Bayesian Networks. *J. Bone Metab.* 25, 251–266. doi: 10.11005/jbm.2018.25.4.251

Peters, J. M., Shah, Y. M., and Gonzalez, F. J. (2012). The role of peroxisome proliferator-activated receptors in carcinogenesis and chemoprevention. *Nat. Rev. Cancer* 12, 181–195. doi: 10.1038/nrc3214

Prat, A., Pineda, E., Adamo, B., Galvan, P., Fernandez, A., Gaba, L., et al. (2015). Clinical implications of the intrinsic molecular subtypes of breast cancer. *Breast* 24 Suppl 2, S26–S35. doi: 10.1016/j.breast.2015.07.008

Rankin, E. B., and Giaccia, A. J. (2016). Hypoxic control of metastasis. *Science* 352, 175–180. doi: 10.1126/science.aaf4405

Rao, J. N., Li, L., Golovina, V. A., Platoshyn, O., Strauch, E. D., Yuan, J. X., et al. (2001). Ca2+-RhoA signaling pathway required for polyamine-dependent intestinal epithelial cell migration. *Am. J. Physiol. Cell Physiol.* 280, C993–1007. doi: 10.1152/ajpcell.2001.280.4.C993

Reddy, J. P., Dawood, S., Mitchell, M., Debeb, B. G., Bloom, E., Gonzalez-Angulo, A. M., et al. (2015). Antiepileptic drug use improves overall survival in breast cancer patients with brain metastases in the setting of whole brain radiotherapy. *Radiother. Oncol.* 117, 308–314. doi: 10.1016/j.radonc.2015.10.009

Remorino, A., De Beco, S., Cayrac, F., Di Federico, F., Cornilleau, G., Gautreau, A., et al. (2017). Gradients of Rac1 Nanoclusters Support Spatial Patterns of Rac1 Signaling. *Cell Rep.* 21, 1922–1935. doi: 10.1016/j.celrep.2017.10.069

Robey, I. F., and Nesbit, L. A. (2013). Investigating mechanisms of alkalinization for reducing primary breast tumor invasion. *BioMed. Res. Int.* 2013, 485196. doi: 10.1155/2013/485196

Roopra, A., Sharling, L., Wood, I. C., Briggs, T., Bachfischer, U., Paquette, A. J., et al. (2000). Transcriptional repression by neuron-restrictive silencer factor is mediated via the Sin3-histone deacetylase complex. *Mol. Cell Biol.* 20, 2147–2157. doi: 10.1128/MCB.20.6.2147-2157.2000

Saint, D. A. (2006). The role of the persistent Na(+) current during cardiac ischemia and hypoxia. J Cardiovasc. *Electrophysiol* 17 Suppl 1, S96–s103. doi: 10.1111/j.1540-8167.2006.00390.x

Selvik, L. K., Rao, S., Steigedal, T. S., Haltbakk, I., Misund, K., Bruland, T., et al. (2014). Salt-inducible kinase 1 (SIK1) is induced by gastrin and inhibits migration of gastric adenocarcinoma cells. *PloS One* 9, e112485. doi: 10.1371/journal.pone.0112485

Sjostrom, M., Stenstrom, K., Eneling, K., Zwiller, J., Katz, A.II, Takemori, H., et al. (2007). SIK1 is part of a cell sodium-sensing network that regulates active sodium transport through a calcium-dependent process. *Proc. Natl. Acad. Sci. U.S.A.* 104, 16922–16927. doi: 10.1073/pnas.0706838104

Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., et al. (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc. Natl. Acad. Sci. U.S.A.* 98, 10869–10874. doi: 10.1073/pnas.191367098

Steffen, A., Ladwein, M., Dimchev, G. A., Hein, A., Schwenkmezger, L., Arens, S., et al. (2013). Rac function is crucial for cell migration but is not required for spreading and focal adhesion formation. *J. Cell Sci.* 126, 4572–4588. doi: 10.1242/jcs.118232

Sumida, G. M., and Yamada, S. (2015). Rho GTPases and the downstream effectors actin-related protein 2/3 (Arp2/3) complex and myosin II induce membrane fusion at self-contacts. *J. Biol. Chem.* 290, 3238–3247. doi: 10.1074/jbc.M114.612168

Sundelacruz, S., Levin, M., and Kaplan, D. L. (2009). Role of membrane potential in the regulation of cell proliferation and differentiation. *Stem Cell Rev. Rep.* 5, 231–246. doi: 10.1007/s12015-009-9080-2

Suraneni, P., Rubinstein, B., Unruh, J. R., Durnin, M., Hanein, D., and Li, R. (2012). The Arp2/3 complex is required for lamellipodia extension and directional fibroblast cell migration. *J. Cell Biol.* 197, 239–251. doi: 10.1083/jcb.201112113

Tran, T. A., Gillet, L., Roger, S., Besson, P., White, E., and Le Guennec, J. Y. (2009). Non-anti-mitotic concentrations of taxol reduce breast cancer cell invasiveness. *Biochem. Biophys. Res. Commun.* 379, 304–308. doi: 10.1016/j.bbrc.2008.12.073

Urbani, A., and Belluzzi, O. (2000). Riluzole inhibits the persistent sodium current in mammalian CNS neurons. *Eur. J. Neurosci.* 12, 3567–3574. doi: 10.1046/j.1460-9568.2000.00242.x

Wannous, R., Bon, E., Maheo, K., Goupille, C., Chamouton, J., Bougnoux, P., et al. (2013). PPARbeta mRNA expression, reduced by n-3 PUFA diet in mammary tumor, controls breast cancer cell growth. *Biochim. Biophys. Acta* 1831, 1618–1625. doi: 10.1016/j.bbalip.2013.07.010

Wannous, R., Bon, E., Gillet, L., Chamouton, J., Weber, G., Brisson, L., et al. (2015). Suppression of PPARbeta, and DHA treatment, inhibit NaV1.5 and NHE-1 pro-invasive activities. *Pflugers Arch.* 467, 1249–1259. doi: 10.1007/s00424-014-1573-4

Xie, H. Y., Shao, Z. M., and Li, D. Q. (2017). Tumor microenvironment: driving forces and potential therapeutic targets for breast cancer metastasis. *Chin. J. Cancer* 36, 36. doi: 10.1186/s40880-017-0202-y

Xu, S. Z., Zeng, F., Lei, M., Li, J., Gao, B., Xiong, C., et al. (2005). Generation of functional ion-channel tools by E3 targeting. *Nat. Biotechnol.* 23, 1289–1293. doi: 10.1038/nbt1148

Xu, X., Zhang, B., Yang, S., An, S., Ribeiro, J. M., and Andersen, J. F. (2016). Structure and Function of FS50, a salivary protein from the flea Xenopsylla cheopis that blocks the sodium channel NaV1.5. *Sci. Rep.* 6, 36574. doi: 10.1038/srep36574

Yamaci, R. F., Fraser, S. P., Battaloglu, E., Kaya, H., Erguler, K., Foster, C. S., et al. (2017). Neonatal Nav1.5 protein expression in normal adult human tissues and breast cancer. *Pathol. Res. Pract.* 213, 900–907. doi: 10.1016/j.prp.2017.06.003

Yang, M., and Brackenbury, W. J. (2013). Membrane potential and cancer progression. *Front. Physiol.* 4, 185. doi: 10.3389/fphys.2013.00185

Yang, X., Wang, D., Dong, W., Song, Z., and Dou, K. (2011). Suppression of Na+/H + exchanger 1 by RNA interference or amiloride inhibits human hepatoma cell line SMMC-7721 cell invasion. *Med. Oncol.* 28, 385–390. doi: 10.1007/s12032-010-9447-x

Yang, M., Kozminski, D. J., Wold, L. A., Modak, R., Calhoun, J. D., Isom, L. L., et al. (2012). Therapeutic potential for phenytoin: targeting Na(v)1.5 sodium channels to reduce migration and invasion in metastatic breast cancer. *Breast Cancer Res. Treat* 134, 603–615. doi: 10.1007/s10549-012-2102-9

Yang, M., James, A. D., Suman, R., Kasprowicz, R., Nelson, M., O'Toole, P. J., et al. (2020). Voltage-dependent activation of Rac1 by Nav 1.5 channels promotes cell migration. *J. Cell Physiol.* 235, 3950–3972. doi: 10.1002/jcp.29290

Yao, W., Feng, D., Bian, W., Yang, L., Li, Y., Yang, Z., et al. (2012). EBP50 inhibits EGF-induced breast cancer cell proliferation by blocking EGFR phosphorylation. *Amino Acids* 43, 2027–2035. doi: 10.1007/s00726-012-1277-z

Yao, P. L., Morales, J. L., Zhu, B., Kang, B. H., Gonzalez, F. J., and Peters, J. M. (2014). Activation of peroxisome proliferator-activated receptor-beta/delta (PPAR-beta/delta) inhibits human breast cancer cell line tumorigenicity. *Mol. Cancer Ther.* 13, 1008–1017. doi: 10.1158/1535-7163.MCT-13-0836

Yeo, S. K., and Guan, J. L. (2017). Breast Cancer: Multiple Subtypes within a Tumor? *Trends Cancer* 3, 753–760. doi: 10.1016/j.trecan.2017.09.001

Yi, M., Huo, L., Koenig, K. B., Mittendorf, E. A., Meric-Bernstam, F., Kuerer, H. M., et al. (2014). Which threshold for ER positivity? a retrospective study based on 9639 patients. *Ann. Oncol.* 25, 1004–1011. doi: 10.1093/annonc/mdu053

Zha, C., Brown, G. B., and Brouillette, W. J. (2014). A highly predictive 3D-QSAR model for binding to the voltage-gated sodium channel: design of potent new ligands. *Bioorg. Med. Chem.* 22, 95–104. doi: 10.1016/j.bmc.2013.11.049

Zhang, B., Deng, Z., Zeng, B., Yang, S., Chen, X., Xu, X., et al. (2018). In-vitro effects of the FS50 protein from salivary glands of Xenopsylla cheopis on voltage-gated sodium channel activity and motility of MDA-MB-231 human breast cancer cells. *Anticancer Drugs* 29, 880–889. doi: 10.1097/CAD.0000000000000662

Zhou, W., and Slingerland, J. M. (2014). Links between oestrogen receptor activation and proteolysis: relevance to hormone-regulated cancer therapy. *Nat. Rev. Cancer* 14, 26–38. doi: 10.1038/nrc3622

Zhou, Y., Wong, C. O., Cho, K. J., van der Hoeven, D., Liang, H., Thakur, D. P., et al. (2015). SIGNAL TRANSDUCTION. Membrane potential modulates plasma membrane phospholipid dynamics and K-Ras signaling. *Science* 349, 873–876. doi: 10.1126/science.aaa5619

Zhu, L., Xi, P. W., Li, X. X., Sun, X., Zhou, W. B., Xia, T. S., et al. (2019). The RNA binding protein RBMS3 inhibits the metastasis of breast cancer by regulating Twist1 expression. *J. Exp. Clin. Cancer Res.* 38, 105. doi: 10.1186/s13046-019-1111-5

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 Luo, Wu, Wu, Chen, Luo, Jiang, Tao, Rong, Kang and Deng. 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

PERSPECTIVE
published: 11 September 2020
doi: 10.3389/fonc.2020.01699

Evaluation of P2X7 Receptor Function in Tumor Contexts Using rAAV Vector and Nanobodies (AAVnano)

*Mélanie Demeules[1], Allan Scarpitta[1], Catalina Abad[1], Henri Gondé[1], Romain Hardet[1], Carolina Pinto-Espinoza[2], Anna Marei Eichhoff[2], Waldemar Schäfer[2], Friedrich Haag[2], Friedrich Koch-Nolte[2] and Sahil Adriouch[1]**

[1] Normandie University, UNIROUEN, INSERM, U1234, Pathophysiology, Autoimmunity, Neuromuscular Diseases and Regenerative THERapies, Rouen, France, [2] Institute of Immunology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

OPEN ACCESS

Edited by:
Lin-Hua Jiang,
University of Leeds, United Kingdom

Reviewed by:
Claudio Coddou,
Catholic University of the North, Coquimbo, Chile
Ben J. Gu,
Florey Institute of Neuroscience and Mental Health, University of Melbourne, Australia

***Correspondence:**
Sahil Adriouch
sahil.adriouch@univ-rouen.fr

Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology

Received: 12 January 2020
Accepted: 30 July 2020
Published: 11 September 2020

Citation:
Demeules M, Scarpitta A, Abad C, Gondé H, Hardet R, Pinto-Espinoza C, Eichhoff AM, Schäfer W, Haag F, Koch-Nolte F and Adriouch S (2020) Evaluation of P2X7 Receptor Function in Tumor Contexts Using rAAV Vector and Nanobodies (AAVnano). Front. Oncol. 10:1699. doi: 10.3389/fonc.2020.01699

Adenosine triphosphate (ATP) represents a danger signal that accumulates in injured tissues, in inflammatory sites, and in the tumor microenvironment. Extracellular ATP is known to signal through plasma membrane receptors of the P2Y and P2X families. Among the P2X receptors, P2X7 has attracted increasing interest in the field of inflammation as well as in cancer. P2X7 is expressed by immune cells and by most malignant tumor cells where it plays a crucial yet complex role that remains to be clarified. P2X7 activity has been associated with production and release of pro-inflammatory cytokines, modulation of the activity and survival of immune cells, and the stimulation of proliferation and migratory properties of tumor cells. Hence, P2X7 plays an intricate role in the tumor microenvironment combining beneficial and detrimental effects that need to be further investigated. For this, we developed a novel methodology termed AAVnano based on the use of Adeno-associated viral vectors (AAV) encoding nanobodies targeting P2X7. We discuss here the advantages of this tool to study the different functions of P2X7 in cancer and other pathophysiological contexts.

Keywords: P2X7, tumor, Adeno-associated virus, Nanobodies (VHH), animal models

INTRODUCTION

Adenosine triphosphate (ATP) released into the extracellular space represents a well-known danger signal that can signal through two main families of plasma membrane receptors: G protein-coupled receptors, named P2Y receptors, and ATP-gated ion channels termed P2X receptors (1). Among the latter family, P2X7 (also known as P2RX7) forms a homotrimeric receptor that has attracted much interest in the fields of inflammation and cancer. Activation of P2X7 by relatively high concentrations of extracellular ATP leads to Na^+ and Ca^{2+} influx, and K^+ efflux. This triggers not only major changes in the cellular ionic content, but also signaling and metabolic pathways involved in cell activation, survival and fate. Prolonged activation of P2X7 leads to the opening of a membrane pore allowing the entry of large molecules of up to 900 Da (2). Whether this membrane permeabilization is due to dilation of the P2X7 channel itself, or the activation of non-selective pores like pannexin-1, gasdermin-D, or anoctamin-6, may depend on the cellular

context, the lipid composition of the membrane, and on the level of expression of these proteins (2–4). Whatever the precise molecular mechanism leading to pore formation, P2X7 activation can lead to major perturbation of intracellular ion balance and to modification of cellular activities, cellular functions, and cell fate.

High concentrations of extracellular ATP, released in the vicinity of stressed or damaged cells, during inflammation, but also within the tumor site, represent a prototypic "danger signal" that can influence the activity and function of immune cells (5–8). P2X7 is expressed at the cell surface of various leukocytes, in particular monocytes, macrophages, T cells and notably regulatory T cells (Tregs), and is found also at the surface of numerous tumor cell types. Extracellular ATP plays a complex role within the tumor microenvironment depending on multiple factors such as its concentration, the abundance of ecto-ATPases, the expression level of P2X7, and the nature of the P2X7 variant expressed by immune and tumor cells (9).

The functions of P2X7 in immune cells are largely documented. Gating P2X7 on macrophage, microglia, and dendritic cells triggers the formation of the inflammasome, a multiprotein complex that drives caspase-mediated maturation and release of the proinflammatory cytokines IL-1β and IL-18 (10). On T lymphocytes, P2X7 induces the shedding of CD62L and CD27 by metalloproteases (11, 12) and controls the differentiation, proliferation and survival of tissue resident memory T cells (13, 14). Regulatory T cells are known to express P2X7 at high levels, and its activation induces shedding of CD62L and CD27, phosphatidylserine exposure, pore formation, and finally leads to cell death (11, 15). Taken together, activation of P2X7 on myeloid and lymphoid cells converge to promote and amplify inflammation (16, 17). Animals deficient for P2X7 show reduced inflammation in several animal models including experimental colitis (18), collagen-induced arthritis (19), and experimental autoimmune encephalomyelitis (EAE) (20). P2X7 receptor antagonists have been developed by several pharmaceutical companies as potential novel anti-inflammatory drugs (21).

ROLE OF P2X7 ON TUMOR GROWTH AND ON ANTI-TUMOR IMMUNE RESPONSES

P2X7 functions in cancer are complex and depend on the composition of the tumor microenvironment and the nature of the cells that express this ion channel. P2X7 is associated with a pro-inflammatory activity which could be beneficial for tumor growth and maintenance. However, P2X7 activation is also linked to immunogenic cell death and has been associated with inflammasome formation and dendritic cell activation, which in turn promote adaptive anti-tumor immune responses (22). As mentioned earlier, P2X7 promotes Treg depletion, which increases antitumor immune responses by unleashing effector functions of T cells (6, 15). In line with the anti-tumor activity of P2X7, tumor cell lines appear to grow faster when transferred to P2X7$^{-/-}$ mice (23), and genetic and pharmacological inactivation of P2X7 increases tumorigenesis in the colitis-associated cancer model (18). However, P2X7 is also expressed by most tumor cells, where it is associated with cancer progression. Indeed, tumor cells express P2X7 at low levels and/or express P2X7 variants that are inefficient to promote cell death but which instead exert a trophic activity. In such situations, tonic stimulation of P2X7 by extracellular ATP already present in the tumor microenvironment can stimulate cell metabolism, influences the Ca^{2+} content of mitochondria and endoplasmic reticulum, increases the intracellular ATP content, protects against apoptosis, and promote tumor growth (24–27). Accordingly, stable transfection of the colon carcinoma tumor model CT26 with a P2X7 expression vector accelerated tumor growth *in vivo* (28), and P2X7 blockade using oxidized ATP (oATP), a poorly selective but irreversible antagonist, reduced melanoma B16 tumor growth *in vivo* (29). Taken together, on the side of the tumor, P2X7 promotes cell survival, tumorigenic potential and proliferation, but on the side of immune cells, P2X7 favors dendritic cell activation, presentation of tumor antigens, and initiation of an adaptive immune response. It appears therefore that the net effect of P2X7 is difficult to predict and that the balance could be tilted toward a pro- or anti-tumorigenic outcome, depending on the composition of the tumor microenvironment, on the level of P2X7 expression, and on the nature and functionality of the P2X7 variants expressed by tumor cells.

METHODS TO STUDY THE ROLE OF P2X7 *IN VIVO*

To investigate the role of P2X7 *in vivo*, several tools have been developed including genetically deficient animals, pharmacological inhibitors, and specific antibodies. P2X7$^{-/-}$ mice have been widely used to study P2X7 function *in vivo* since the generation of the first two P2X7 KO strains by Pfizer and Glaxo (30, 31). However, it subsequently became apparent that both lines are leaky: the Glaxo line, in which exon 1 was targeted, still expresses the P2X7k variant on T cells (32, 33) while the Pfizer line, in which exon 13 was targeted, still expresses a C-terminal truncated variant displaying lower functionality (34). This situation could explain reported phenotypic and functional differences as well as conflicting results in disease models using these two P2X7 deficient lines (20, 35, 36). However, novel knockout models and P2X7-floxed mutants derived from the European Mutant Mouse Archive (EMMA) are now available and may facilitate the reevaluation of P2X7 functions *in vivo* in different disease models (37, 38).

Pharmacological inhibitors of P2X7 like brilliant blue G (BBG) and oATP have shown therapeutic benefit in several animal models including EAE, experimental colitis, inflammatory pain in arthritic animals, or/and antibody-mediated nephritis (36, 39–42). Blocking P2X7 by these small molecule inhibitors has also been shown to inhibit tumor growth in several tumor models that express P2X7 (28, 29). BBG and oATP are rather inexpensive but lack specificity and are associated with off-target side effects. The development of more specific antagonists by several pharmaceutical companies has facilitated the evaluation

and confirmation of the role of P2X7 in these diseases (23, 28, 36, 39). However, these antagonists are sometimes more difficult to obtain and are expensive to use, notably in chronic models where they have to be injected every other days for several weeks.

P2X7 MODULATING NANOBODIES

Antibodies represent another emerging class of potent pharmaceutical modulators that are used to block or to potentiate their targets *in vivo*. Conventional antibodies are composed of two heavy and two light chains that contribute together to the antigen-binding site. Conventional antibodies represent relatively large molecules of ∼150 kDa. In direct relation with their size, their biodistribution into tissues is limited (43). In comparison, smaller proteins derived from antibodies but containing only the binding domains fused directly via a linker (i.e., single-chain variable fragment or scFv) offer better biodistribution coefficients. More recently developed nanobodies offer an interesting alternative. Nanobodies are derived from unconventional antibodies found in llamas and other camelids that are composed only of heavy chains. Nanobodies and heavy chain antibodies present similar specificities and affinities as classical antibodies but exhibit a smaller size (80 kDa, 15 kDa) and a better tissue penetration notably into tumors (44–46).

A set of nanobodies modulating P2X7 was selected by phage display from llamas immunized with cDNA expression vectors or P2X7-transfected HEK cells encoding for mouse and human P2X7 (47). In this study, two nanobodies were selected and used to modulate the function of mouse P2X7. Interestingly, one of them potently blocks P2X7 while the other potentiates its activities. These nanobodies, termed 13A7 and 14D5, respectively, were used *in vivo* to validate the function of P2X7 in disease models. Systemic administration of 13A7 reduced inflammation in mouse models of allergic dermatitis and of glomerulonephritis. Conversely, 14D5 administrated *in vivo* aggravated disease scores in both animal models (47). Repeated administration of these selected anti-P2X7 nanobodies *in vivo* undoubtedly represents a novel means to study P2X7 functions in different pathophysiological situations.

AAV-NANOBODIES (AAVnano) METHODOLOGY

We present and illustrate here the development of a novel methodology using adeno-associated viral vectors (AAV) encoding anti-P2X7 nanobodies for studying P2X7 function *in vivo,* notably in chronic situations. AAV vectors have widely been used for gene transfer *in vivo* to elicit long-term expression of the transgenic protein of interest. For instance, a single intramuscular injection of AAV encoding HIV-neutralizing antibodies resulted in their long-lasting systemic production in mice (48) and non-human primates (49). We implemented a similar AAV-mediated gene transfer method to produce *in situ* anti-P2X7 nanobodies with the aim to durably modulate P2X7 function *in vivo*. For that, we generated AAV vectors encoding a bivalent P2X7-antagonistic heavy chain antibody designated 13A7-hcAb (nanobody 13A7 fused to the hinge and Fc-domains of mouse IgG1) or a bivalent, half-life extended P2X7-potentiating nanobody dimer designated 14D5-dimHLE (dimer of nanobody 14D5 fused to the albumin-specific nanobody Alb8 conferring half-life extension) (47). The methodology that we developed, termed AAVnano, requires only a single intramuscular (i.m.) injection of the corresponding AAV vector to elicit long-term systemic expression of these nanobody constructs *in vivo* for at least 120 days (unpublished observations). This avoids the daily injection of nanobodies necessary to maximize the modulation of P2X7 functions and offers the possibility to inhibit or to potentiate P2X7 in chronic models such as chronic inflammation, autoimmune diseases, carcinogenesis, or tumor growth (**Figure 1**).

ILLUSTRATION OF THE AAVnano METHODOLOGY IN A TUMOR MODEL

As discussed earlier, P2X7 plays complex and partially opposing roles in tumor growth and anti-tumor immune responses. To evaluate the potential utility of the AAVnano methodology, we chose to study the *in vivo* growth of the C57BL/6 mouse lymphoma cell line EG7 that naturally expresses significant surface levels of P2X7 (**Figure 2A**). We found that EG7 naturally expresses P2X7 at a level that is similar to the level detected at the surface of A20-P2X7 cells obtained after stable transfection of the BALB/c mouse B cell lymphoma A20 with an expression plasmid encoding full-length mouse P2X7 (50). To further characterize the functionality of P2X7 on EG7 cells, we verified their sensitivity to cell death induced by high concentrations of ATP (**Figure 2B**). As expected, addition of the recombinant nanobodies 13A7-hcAb or 14D5-dimHLE, respectively, inhibited and potentiated ATP induced-cell death, confirming the involvement and the functionality of P2X7 (**Figure 2C**).

Next, we aimed to study the situation *in vivo* in a context where P2X7 would be permanently and durably blocked or potentiated by 13A7-hcAb or 14D5-dimHLE nanobodies produced *in situ*. For that, AAV1 vectors encoding these constructs were injected intramuscularly (i.m.) into syngeneic naive C57BL/6 mice 3 weeks before inoculation of tumor cells. Before tumor inoculation, we obtained blood samples from the AAV injected mice. In order to evaluate the functional effects of nanobodies bound to circulating T cells *in vivo*, we incubated blood leukocytes obtained from these mice with ATP *ex vivo* and analyzed their sensitivity to ATP-induced shedding of CD27 and CD62L, a sensitive P2X7-dependent effect (15). As expected, $CD4^+$ T lymphocytes from mice injected with AAV1 encoding P2X7-antagonistic 13A7-hcAb were resistant to ATP-induced CD27 and CD62L shedding while cells from the mice injected with AAV1 encoding the P2X7-potentiating 14D5-dimHLE showed enhanced sensitivity to ATP-induced shedding of CD27 and CD62L (**Figure 2D**). Further, indirect flow cytometry analyses *ex vivo* of EG7 and A20-P2X7 cells with

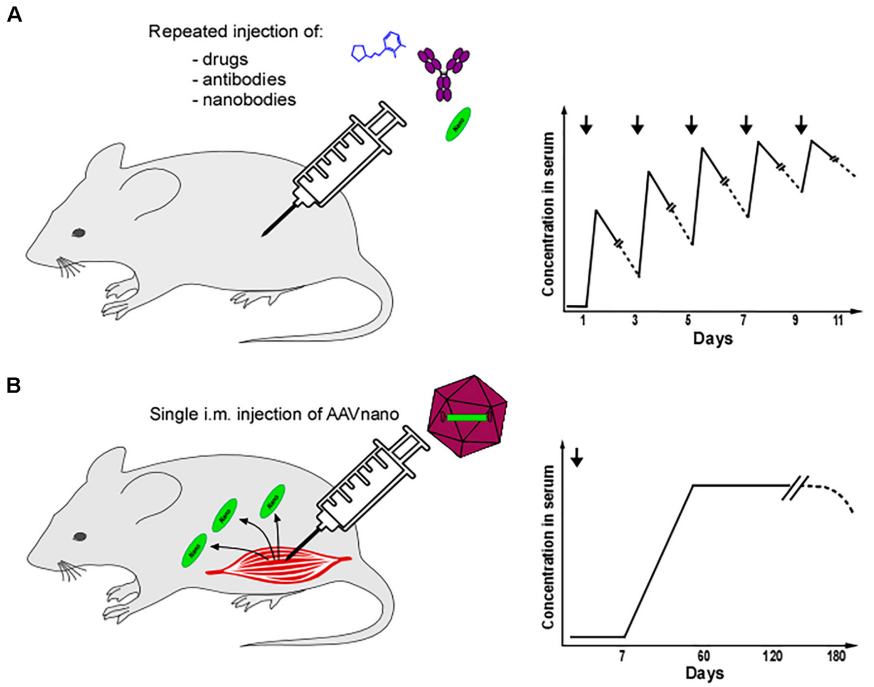


FIGURE 1 | Comparison between repeated injection of drugs or antibodies/nanobodies and AAVnano methodology. Theoretical kinetics of the pharmacological agents in serum using different methodologies. **(A)** Small molecules (blue), antibodies (purple), or nanobodies (green) are generally injected using the i.p. or i.v. routes and necessitate repeated injections usually performed every 1–2 days. **(B)** Using the AAVnano methodology, a single injection of an AAV vector coding for the nanobody of interest is performed at day 0 using the i.m. route. The nanobody is then directly produced *in vivo* by the transduced muscle fibers. The nanobody is detectable in the serum 7–14 days post AAV injection and its concentration slowly increases until reaching a plateau 30–60 days post AAV injection. The nanobody produced *in vivo* can still be present in the serum 120 days post AAV injection.

plasma from these animals confirmed the presence of circulating nanobodies 3 weeks after AAV injection (**Figure 2E**).

Next, the EG7 tumor cells were injected subcutaneously, and tumor growth was monitored for 50 days. The results show that constant production of the P2X7 antagonistic 13A7-hcAb significantly limited EG7 tumor growth *in vivo* (**Figure 2F**). This treatment induced a complete tumor regression in 2 out of 5 mice and significantly improved the survival of treated mice (**Figure 2G**). These data are in line with previous studies showing that expression of P2X7 by tumor cells represents a trophic and pro-survival factor and that P2X7-blockade can inhibit tumor growth *in vivo* (24, 25, 28, 29). In contrast, potentiation of P2X7 function using AAV-mediated *in situ* expression of 14D5-dimHLE did not significantly influence tumor growth in this model (**Figures 2F,G**). One might have expected the opposite effect than 13A7-hcAb, e.g., promotion of tumor growth. On the other hand, P2X7 potentiation could also promote anti-tumor immune responses by mediating death of regulatory T cells (Tregs) by enhancing their sensitivity to NAD-mediated cell death (NICD). We indeed previously demonstrated that P2X7-dependent NICD of Tregs promotes an anti-tumor immune response in the same tumor model (15). The lack of an effect of 14D5-DimHLE on tumor growth could thus reflect balanced anti-tumoral/pro-tumoral effects in the tumor microenvironment. It will be interesting to determine whether potentiation of P2X7 can enhance anti-tumor immune response when combined with other treatments, e.g., anti-immune checkpoints therapy, immunogenic chemotherapies that increase the release of ATP, or antagonism of CD39/CD73-catalyzed hydrolysis of ATP to immunosuppressive adenosine (51, 52).

Overall, our data support the notion that the AAVnano methodology represents an additional strategy to study the function of P2X7 *in vivo,* allowing target validation in pathophysiological situations where P2X7 has been implicated, including cancer.

DISCUSSION AND PERSPECTIVES OFFERED BY THE AAVnano METHODOLOGY TO STUDY THE IMPORTANCE OF P2X7 IN DIFFERENT PATHOPHYSIOLOGICAL SITUATIONS

The AAVnano methodology presents several theoretical and practical advantages over other methodologies. As compared to chemical compounds, the nanobodies offer better target specificity. Indeed, nanobodies and antibodies in general are known for their excellent ability to discriminate between closely related targets belonging to the same protein family. This may prevent unwanted off-target effects. In the present study, the

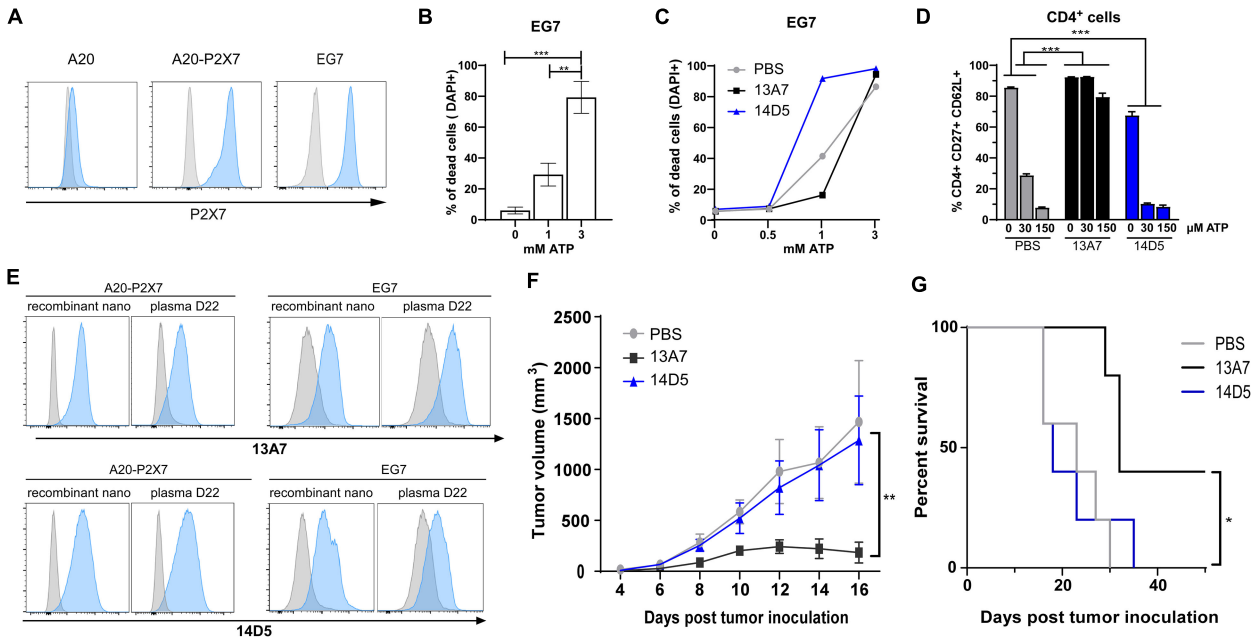


FIGURE 2 | Using the AAVnano methodology to study the role of P2X7 in the EG7 tumor model. **(A)** C57BL/6 mouse EG7 T lymphoma cells expresses P2X7 at the cell membrane. EG7 T lymphoma cells, A20 B lymphoma cells and A20 cells stably transfected with P2X7 were incubated with fluorochrome conjugated P2X7-specific mAb Hano43 (blue) or an isotype control (gray). Bound antibodies were detected by flow cytometry. Similar results were obtained using the P2X7 specific polyclonal antibodies K1G (58). **(B)** EG7 cells are sensitive to ATP-induced cell death *in vitro*. EG7 cells were incubated overnight in culture medium with the indicated concentrations of ATP. Cell death was assessed by flow cytometry after staining with DAPI. The statistical analysis were performed using one-way ANOVA ($n = 3$, $^{**}p < 0.01$; $^{***}p < 0.001$). **(C)** P2X7-specific nanobodies modulate ATP-induced death of EG7 cells. EG7 cells were preincubated for 1 h in the absence (PBS, gray) or presence of 1 µg/ml 13A7-hcAb (13A7, black) or 14D5-dimHLE (14D5, blue). Cells were then incubated overnight with 0, 0.5, 1, or 3 mM of ATP in culture medium and assayed for cell death using flow cytometry as in **(B)**. **(D)** Nanobodies produced *in situ* modulate P2X7-dependent shedding of CD62L and CD27 by $CD4^+$ T cells. 8 weeks old C57BL/6 mice were injected i.m. with PBS (gray) or with AAV1 vector encoding either P2X7-antagonistic 13A7-hcAb (black) or P2X7-potentiating 14D5-dimHLE (blue). 3 weeks later (day 22), blood samples were collected and incubated for 20 min in PBS with the indicated concentration of ATP (0, 30, or 150 µM ATP). Cells were then stained with fluorochrome-conjugated antibodies against CD4, CD27, CD62L, before fixation and erythrocyte lysis. The percentages of $CD4^+$ cells that co-express CD27 and CD62L were then evaluated by flow cytometry. The statistical analysis were performed using two-way ANOVA ($n = 5$, $^{***}p < 0.001$). Similar results were obtained in 3 independent experiments. **(E)** Plasma from AAV-injected mice contain circulating P2X7-specific nanobodies. A20-P2X7 and EG7 cells were incubated either with recombinant nanobodies (1 µg/ml) or with 10 µl of pooled plasma obtained at day 22 after AAV injection. Bound nanobodies were detected by flurochrome-conjugated secondary antibodies using flow cytometry. Histograms in gray correspond to the background staining obtained when using the sera from PBS injected animals. **(F,G)** P2X7 antagonist but not P2X7-agonist constructs inhibit EG7 lymphoma tumor growth *in vivo*. C57BL/6 mice were injected i.m. with 10^{11} AAV1 vectors encoding the P2X7-antagonistic 13A7-hcAb (13A7, black line), or the P2X7-potentiating 14D5-dimHLE (14D5, blue line). 23 days later, after having confirmed the production and the functionality of these constructs produced *in vivo* (see above **D,E**), 10^6 EG7 lymphoma cells were inoculated subcutaneously in the left flanks. Tumors were monitored every other day during 50 days with a digital caliper, and the tumor volumes were estimated using the formula length × width × [(length + width)/2]. **(F)** Tumor volumes in each experimental group are shown for the first 16 days. The statistical analysis were performed using two-way ANOVA ($n = 5$, $^{**}p < 0.01$). **(G)** Mouse survival is illustrated during the entire observation period. The statistical analysis was performed using Log-Rank tests ($n = 5$, $^{*}p < 0.05$). Similar results were obtained in 3 independent experiments.

selected nanobodies bind specifically to mouse P2X7 and do not recognize the closest paralogs P2X4 and P2X1 (47). However, high specificity and lack of cross-reactivity with the P2X7 ortholog of other species may limit the use of a nanobody across species in translational studies. A similar limitation has also been observed with chemical compounds that sometimes display different modulating effects and pharmacological proprieties in humans and other animals.

Adeno-associated viral vectors are widely used in gene therapy settings and represent an efficient and safe approach to transfer a gene of interest into muscle cells and to elicit long-term systemic *in situ* production of a transgenic protein (48, 53). In our experiences, a single intramuscular administration of AAV (10^{11} viral genomes per mouse) coding for our engineered nanobody constructs was sufficient to elicit effective systemic levels of the blocking or the potentiating P2X7-specific nanobodies. The AAVnano methodology thus avoids daily administration of the corresponding recombinant nanobodies. Arguably, this may additionally provide more favorable pharmacokinetics by maintaining a stable saturating concentration of the desired nanobody *in vivo* and by avoiding peak/decline cycles obtained after repeated injection of a recombinant antibody (**Figure 1**). This may favor a better bioavailability and tissue penetration, which needs to be confirmed experimentally in future studies. AAVnano may provide a nanobody delivery methodology similar to the one obtained with osmotic pump drug delivery systems, which allow a stable equilibrium of the pharmacological agent

in vivo at a plateau phase (**Figure 1**). However, as systemic production of transgenic protein *in situ* using AAV vectors is known to follow slow kinetics, with the apparition of the protein of interest 1 to 2 weeks post-transduction, the equilibrium phase is probably not obtained *in vivo* before 2 to 3 weeks (54–56). Hence, the AAVnano methodology is intended to be used in prophylactic models, as in the present study, rather than in a therapeutic setting. However, the kinetics of transgenic protein production can be considerably accelerated using recently developed self-complementary AAV (scAAV) vectors that would be more compatible with therapeutic/interventional protocols (56).

One of the features associated with the AAVnano methodology is the permanent and long-term modulation of P2X7 *in vivo* without any possibility to halt the treatment. While this is adapted to the evaluation of P2X7 functions in chronic diseases, this may appear as an inconvenient in other situations. Additionally, long-term P2X7 modulation (i.e., activation or potentiation) may have unexpected effects on the general physiology of the mice by causing, for instance, immune dysregulation, alteration of barrier functions, or alteration of the microbiota. Knockout mice may possibly not be affected to the same extent by P2X7 deficiency as compensatory mechanisms are presumably induced during embryogenesis, as often observed in knockout animals. In this regard, AAVnano methodology may reveal so far unexpected physiological functions of P2X7 that may be interesting to further explore. One way to avoid such long-term effects would be to use inducible promoters, instead of the ubiquitous CBA promoter used in this study. Such an inducible system, combined with AAV-mediated gene transfer, would allow controlled and timely delivery of the selected nanobody to modulate P2X7 only when required. This type of inducible delivery would be compatible with both, a prophylactic scheme as well as therapeutic/interventional protocols.

Beyond the cancer field, the AAVnano methodology may be further exploited in target-validation studies and in other disease models where P2X7 has been incriminated. Examples are inflammatory models such as acute and chronic experimental colitis, or autoimmune diseases like rheumatoid arthritis, lupus, or experimental allergic encephalitis. Interestingly, besides skeletal muscles, AAV vectors can be used to transduce efficiently a variety of other tissues including heart, liver, kidney, retina, and the central nervous system (CNS). For example, it is conceivable to use stereotaxic injection of AAV9 vectors into the brain to induce expression of the modulating P2X7-specific nanobodies in the CNS. Since P2X7 is expressed in the CNS, particularly by microglial cells and astrocytes, AAVnano may help to elucidate the role of P2X7 in the pathophysiology of neurological disorders like epilepsy, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis, age-related macular degeneration, or cerebral artery occlusion (57). Apart from using tissue-specific conditional P2X7 knockout models, the role of P2X7 in these diseases remains difficult to evaluate as the use of antibodies is limited by the blood-brain barrier. AAVnano may offer a possibility to circumvent this difficulty by exploiting the efficiency of AAV vectors to transduce different regions of the CNS, combined with the good tissue penetration and bioavailability offered by nanobodies. Hence, the AAVnano methodology may pave the way to a better understanding of the role of P2X7 not only in the tumor context, but also in chronic inflammatory/autoimmune diseases and neurological disorders. This may further help the community to evaluate the importance of P2X7 in pathophysiology and as a therapeutic target.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité d'Ethique NOrmandie en Matière d'EXpérimentation Animale (CENOMEXA).

AUTHOR CONTRIBUTIONS

MD, FH, FK-N, and SA conceptualization. MD, AS, CA, and SA methodology. MD, CA, and SA investigation. MD, FK-N, and SA writing – original draft. MD, AS, CA, HG, RH, CP-E, AE, WS, FH, FK-N, and SA writing – review and editing. FK-N and SA funding acquisition. FH, FK-N, and SA Resources. CA, FK-N, and SA supervision. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG) to FK-N (310/13-1) and by a grant from the Agence Nationale de la Recherche (ANR) to SA (ANR-18-CE92-0046).

REFERENCES

1. Burnstock G. Purinergic signalling. *Br J Pharmacol.* (2009) 147:S172–81. doi: 10.1038/sj.bjp.0706429
2. Di Virgilio F, Schmalzing G, Markwardt F. The elusive P2X7 macropore. *Trends Cell Biol.* (2018) 28:392–404. doi: 10.1016/j.tcb.2018.01.005
3. Di Virgilio F, Giuliani AL, Vultaggio-Poma V, Falzoni S, Sarti AC. Non-nucleotide agonists triggering P2X7 receptor activation and pore formation. *Front Pharmacol.* (2018) 9:39. doi: 10.3389/fphar.2018.00039
4. Peverini L, Beudez J, Dunning K, Chataigneau T, Grutter T. New insights into permeation of large cations through ATP-gated P2X receptors. *Front Mol Neurosci.* (2018) 11:265. doi: 10.3389/fnmol.2018.00265

5. Adriouch S, Haag F, Boyer O, Seman M, Koch-Nolte F. Extracellular NAD(+): a danger signal hindering regulatory T cells. *Microbes Infect*. (2012) 14:1284–92. doi: 10.1016/j.micinf.2012.05.011
6. Rissiek B, Haag F, Boyer O, Koch-Nolte F, Adriouch S. ADP-ribosylation of P2X7: a matter of life and death for regulatory T cells and natural killer T cells. *Curr Top Microbiol Immunol*. (2015) 384:107–26. doi: 10.1007/82_2014_420
7. Gilbert S, Oliphant C, Hassan S, Peille A, Bronsert P, Falzoni S, et al. ATP in the tumour microenvironment drives expression of nfP2X 7, a key mediator of cancer cell survival. *Oncogene*. (2019) 38:194–208. doi: 10.1038/s41388-018-0426-6
8. Pellegatti P, Raffaghello L, Bianchi G, Piccardi F, Pistoia V, Di Virgilio F. Increased level of extracellular ATP at tumor sites: In vivo imaging with plasma membrane luciferase. *PLoS One*. (2008) 3:e2599. doi: 10.1371/journal.pone.0002599
9. Young CNJ, Górecki DC. P2RX7 purinoceptor as a therapeutic target-the second coming? *Front Chem*. (2018) 6:248. doi: 10.3389/fchem.2018.00248
10. Perregaux DG, McNiff P, Laliberte R, Conklyn M, Gabel CA. ATP acts as an agonist to promote stimulus-induced secretion of IL-1 beta and IL-18 in human blood. *J Immunol*. (2000) 165:4615–23. doi: 10.4049/jimmunol.165.8.4615
11. Rissiek B, Haag F, Boyer O, Koch-Nolte F, Adriouch S. P2X7 on mouse T cells: one channel, many functions. *Front Immunol*. (2015) 6:204. doi: 10.3389/fimmu.2015.00204
12. Sommer A, Kordowski F, Büch J, Maretzky T, Evers A, Andrä J, et al. Phosphatidylserine exposure is required for ADAM17 sheddase function. *Nat Commun*. (2016) 7:11523. doi: 10.1038/ncomms11523
13. Stark R, Wesselink TH, Behr FM, Kragten NAM, Arens R, Koch-Nolte F, et al. TRM maintenance is regulated by tissue damage via P2RX7. *Sci Immunol*. (2018) 3:eaau1022. doi: 10.1126/SCIIMMUNOL.AAU1022
14. Rissiek B, Lukowiak M, Raczkowski F, Magnus T, Mittrücker HW, Koch-Nolte F. Blockade of murine ARTC2.2 during cell preparation preserves the vitality and function of liver tissue-resident memory T cells. *Front Immunol*. (2018) 9:1580. doi: 10.3389/fimmu.2018.01580
15. Hubert S, Rissiek B, Klages K, Huehn J, Sparwasser T, Haag F, et al. Extracellular NAD+ shapes the Foxp3+ regulatory T cell compartment through the ART2-P2X7 pathway. *J Exp Med*. (2010) 207:2561–8. doi: 10.1084/jem.20091154
16. Di Virgilio F, Dal Ben D, Sarti AC, Giuliani AL, Falzoni S. The P2X7 receptor in infection and inflammation. *Immunity*. (2017) 47:15–31. doi: 10.1016/j.immuni.2017.06.020
17. Adinolfi E, Giuliani AL, De Marchi E, Pegoraro A, Orioli E, Di Virgilio F. The P2X7 receptor: a main player in inflammation. *Biochem Pharmacol*. (2018) 151:234–44. doi: 10.1016/j.bcp.2017.12.021
18. Hofman P, Cherfils-Vicini J, Bazin M, Ilie M, Juhel T, Hébuterne X, et al. Genetic and pharmacological inactivation of the purinergic P2RX7 receptor dampens inflammation but increases tumor incidence in a mouse model of colitis-associated cancer. *Cancer Res*. (2015) 75:835–45. doi: 10.1158/0008-5472.CAN-14-1778
19. Labasi JM, Petrushova N, Donovan C, McCurdy S, Lira P, Payette MM, et al. Absence of the P2X 7 receptor alters leukocyte function and attenuates an inflammatory response. *J Immunol*. (2002) 168:6436–45. doi: 10.4049/jimmunol.168.12.6436
20. Sharp AJ, Polak PE, Simonini V, Lin SX, Richardson JC, Bongarzone ER, et al. P2x7 deficiency suppresses development of experimental autoimmune encephalomyelitis. *J Neuroinflammation*. (2008) 5:33. doi: 10.1186/1742-2094-5-33
21. Park J-H, Kim Y-C. P2X7 receptor antagonists: a patent review. (2010–2015). *Expert Opin Ther Pat*. (2017) 27:257–67. doi: 10.1080/13543776.2017.1246538
22. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, et al. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1β–dependent adaptive immunity against tumors. *Nat Med*. (2009) 15:1170–8. doi: 10.1038/nm.2028
23. Adinolfi E, Capece M, Franceschini A, Falzoni S, Giuliani AL, Rotondo A, et al. Accelerated tumor progression in mice lacking the ATP receptor P2X7. *Cancer Res*. (2015) 75:635–44. doi: 10.1158/0008-5472.CAN-14-1259
24. Adinolfi E, Callegari MG, Ferrari D, Bolognesi C, Minelli M, Wieckowski MR, et al. Basal activation of the P2X7 ATP receptor elevates mitochondrial calcium and potential, increases cellular ATP levels, and promotes serum-independent growth. *Mol Biol Cell*. (2005) 16:3260–72. doi: 10.1091/mbc.e04-11-1025
25. Adinolfi E, Callegari MG, Cirillo M, Pinton P, Giorgi C, Cavagna D, et al. Expression of the P2X7 receptor increases the Ca2+ content of the endoplasmic reticulum, activates NFATc1, and protects from apoptosis. *J Biol Chem*. (2009) 284:10120–8. doi: 10.1074/jbc.M805805200
26. Roger S, Jelassi B, Couillin I, Pelegrin P, Besson P, Jiang L-H. Understanding the roles of the P2X7 receptor in solid tumour progression and therapeutic perspectives. *Biochim Biophys Acta*. (2015) 1848:2584–602. doi: 10.1016/j.bbamem.2014.10.029
27. Roger S, Pelegrin P. P2X7 receptor antagonism in the treatment of cancers. *Expert Opin Investig Drugs*. (2011) 20:875–80. doi: 10.1517/13543784.2011.583918
28. Adinolfi E, Raffaghello L, Giuliani AL, Cavazzini L, Capece M, Chiozzi P, et al. Expression of P2X7 receptor increases in vivo tumor growth. *Cancer Res*. (2012) 72:2957–69. doi: 10.1158/0008-5472.CAN-11-1947
29. Hattori F, Ohshima Y, Seki S, Tsukimoto M, Sato M, Takenouchi T, et al. Feasibility study of B16 melanoma therapy using oxidized ATP to target purinergic receptor P2X7. *Eur J Pharmacol*. (2012) 695:20–6. doi: 10.1016/j.ejphar.2012.09.001
30. Solle M, Labasi J, Perregaux DG, Stam E, Petrushova N, Koller BH, et al. Altered cytokine production in mice lacking P2X(7) receptors. *J Biol Chem*. (2001) 276:125–32. doi: 10.1074/jbc.M006781200
31. Chessell IP, Hatcher JP, Bountra C, Michel AD, Hughes JP, Green P, et al. Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory and neuropathic pain. *Pain*. (2005) 114:386–96. doi: 10.1016/j.pain.2005.01.002
32. Nicke A, Kuan YH, Masin M, Rettinger J, Marquez-Klaka B, Bender O, et al. A functional P2X7 splice variant with an alternative transmembrane domain 1 escapes gene inactivation in P2X7 knock-out mice. *J Biol Chem*. (2009) 284:25813–22. doi: 10.1074/jbc.M109.033134
33. Taylor SR, Gonzalez-Begne M, Sojka DK, Richardson JC, Sheardown SA, Harrison SM, et al. Lymphocytes from P2X7-deficient mice exhibit enhanced P2X7 responses. *J Leukoc Biol*. (2009) 85:978–86. doi: 10.1189/jlb.0408251
34. Masin M, Young C, Lim K, Barnes SJ, Xu XJ, Marschall V, et al. Expression, assembly and function of novel C-terminal truncated variants of the mouse P2X7 receptor: re-evaluation of P2X7 knockouts. *Br J Pharmacol*. (2012) 165:978–93. doi: 10.1111/j.1476-5381.2011.01624.x
35. Chen L, Brosnan CF. Exacerbation of experimental autoimmune encephalomyelitis in P2X7R-/- mice: evidence for loss of apoptotic activity in lymphocytes. *J Immunol*. (2006) 176:3115–26. doi: 10.4049/jimmunol.176.5.3115
36. Bartlett R, Stokes L, Sluyter R. The P2X7 receptor channel: recent developments and the use of P2X7 antagonists in models of disease. *Pharmacol Rev*. (2014) 66:638–75. doi: 10.1124/pr.113.008003
37. Kaczmarek-Hajek K, Zhang J, Kopp R, Grosche A, Rissiek B, Saul A, et al. Re-evaluation of neuronal P2X7 expression using novel mouse models and a P2X7-specific nanobody. *Elife*. (2018) 7:e36217. doi: 10.7554/eLife.36217
38. Faliti CE, Gualtierotti R, Rottoli E, Gerosa M, Perruzza L, Romagnani A, et al. P2X7 receptor restrains pathogenic Tfh cell generation in systemic lupus erythematosus. *J Exp Med*. (2019) 216:317–36. doi: 10.1084/jem.20171976
39. Arulkumaran N, Unwin RJ, Tam FW. A potential therapeutic role for P2X7 receptor (P2X7R) antagonists in the treatment of inflammatory diseases. *Expert Opin Investig Drugs*. (2011) 20:897–915. doi: 10.1517/13543784.2011.578068
40. Matute C, Torre I, Pérez-Cerdá F, Pérez-Samartín A, Alberdi E, Etxebarria E, et al. P2X(7) receptor blockade prevents ATP excitotoxicity in oligodendrocytes and ameliorates experimental autoimmune encephalomyelitis. *J Neurosci*. (2007) 27:9525–33. doi: 10.1523/JNEUROSCI.0579-07.2007
41. Dell'Antonio G, Quattrini A, Dal Cin E, Fulgenzi A, Ferrero ME. Antinociceptive effect of a new P(2Z)/P2X7 antagonist, oxidized ATP, in arthritic rats. *Neurosci Lett*. (2002) 327:87–90. doi: 10.1016/s0304-3940(02)00385-3

42. Taylor SR, Turner CM, Elliott JI, McDaid J, Hewitt R, Smith J, et al. P2X7 deficiency attenuates renal injury in experimental glomerulonephritis. *J Am Soc Nephrol*. (2009) 20:1275–81. doi: 10.1681/ASN.2008060559
43. Li Z, Krippendorff BF, Sharma S, Walz AC, Lavé T, Shah DK. Influence of molecular size on tissue distribution of antibody fragments. *MAbs*. (2016) 8:113–9. doi: 10.1080/19420862.2015.1111497
44. Wesolowski J, Alzogaray V, Reyelt J, Unger M, Juarez K, Urrutia M, et al. Single domain antibodies: promising experimental and therapeutic tools in infection and immunity. *Med Microbiol Immunol*. (2009) 198:157–74. doi: 10.1007/s00430-009-0116-7
45. Muyldermans S. Nanobodies: natural single-domain antibodies. *Annu Rev Biochem*. (2013) 82:775–97. doi: 10.1146/annurev-biochem-063011-092449
46. Bannas P, Hambach J, Koch-Nolte F. Nanobodies and nanobody-based human heavy chain antibodies as antitumor therapeutics. *Front Immunol*. (2017) 8:1603. doi: 10.3389/fimmu.2017.01603
47. Danquah W, Catherine MS, Rissiek B, Pinto C, Arnau SP, Amadi M, et al. Nanobodies that block gating of the P2X7 ion channel ameliorate inflammation. *Sci Transl Med*. (2016) 8:366ra162. doi: 10.1126/scitranslmed.aaf8463
48. Balazs AB, Ouyang Y, Hong CM, Chen J, Nguyen SM, Rao DS, et al. Vectored immunoprophylaxis protects humanized mice from mucosal HIV transmission. *Nat Med*. (2014) 20:296–300. doi: 10.1038/nm.3471
49. Saunders KO, Wang L, Gordon Joyce M, Yang Z-Y, Balazs AB, Cheng C, et al. Broadly neutralizing human immunodeficiency virus type 1 antibody gene transfer protects non-human primates from mucosal simian-human immunodeficiency virus infection. *J Virol*. (2015) 89:8334–45. doi: 10.1128/JVI.00908-15
50. Adriouch S, Bannas P, Schwarz N, Fliegert R, Guse AH, Seman M, et al. ADP−ribosylation at R125 gates the P2X7 ion channel by presenting a covalent ligand to its nucleotide binding site. *FASEB J*. (2008) 22:861–9. doi: 10.1096/fj.07-9294com
51. Garg AD, Krysko DV, Vandenabeele P, Agostinis P. Extracellular ATP and P2X7 receptor exert contextspecific immunogenic effects after immunogenic cancer cell death. *Cell Death Dis*. (2016) 7:e2097. doi: 10.1038/cddis.2015.411
52. Hammami A, Allard D, Allard B, Stagg J. Targeting the adenosine pathway for cancer immunotherapy. *Semin Immunol*. (2019) 42:101304. doi: 10.1016/j.smim.2019.101304
53. Saunders KO, Wang L, Joyce MG, Yang ZY, Balazs AB, Cheng C, et al. Broadly neutralizing human immunodeficiency virus type 1 antibody gene transfer protects non-human primates from mucosal simian-human immunodeficiency virus infection. *J Virol*. (2015) 89:8334–45.
54. Adriouch S, Franck E, Drouot L, Bonneau C, Jolinon N, Salvetti A, et al. Improved immunological tolerance following combination therapy with CTLA-4/Ig and AAV-mediated PD-L1/2 muscle gene transfer. *Front Microbiol*. (2011) 2:199. doi: 10.3389/fmicb.2011.00199
55. Hardet R, Chevalier B, Dupaty L, Naïmi Y, Riou G, Drouot L, et al. Oral-tolerization prevents immune responses and improves transgene persistence following gene transfer mediated by adeno-associated viral vector. *Mol Ther*. (2016) 24:87–95. doi: 10.1038/mt.2015.146
56. McCarty DM. Self-complementary AAV vectors; advances and applications. *Mol Ther*. (2008) 16:1648–56. doi: 10.1038/mt.2008.171
57. Kanellopoulos JM, Delarasse C. Pleiotropic roles of P2X7 in the central nervous system. *Front Cell Neurosci*. (2019) 13:401. doi: 10.3389/fncel.2019.00401
58. Seman M, Adriouch S, Scheuplein F, Krebs C, Freese D, Glowacki G, et al. NAD-induced T cell death: ADP-ribosylation of cell surface proteins by ART2 activates the cytolytic P2X7 purinoceptor. *Immunity*. (2003) 19:571–82. doi: 10.1016/S1074-7613(03)00266-8

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 © 2020 Demeules, Scarpitta, Abad, Gondé, Hardet, Pinto-Espinoza, Eichhoff, Schäfer, Haag, Koch-Nolte and Adriouch. 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 Pharmacology

REVIEW
published: 29 September 2020
doi: 10.3389/fphar.2020.581455

TRP Channels and Small GTPases Interplay in the Main Hallmarks of Metastatic Cancer

Giorgia Chinigò[1,2], *Alessandra Fiorio Pla*[1,2] *and Dimitra Gkika*[2,3,4]*

[1] Laboratory of Cellular and Molecular Angiogenesis, Department of Life Sciences and Systems Biology, University of Torino, Torino, Italy, [2] Laboratoire de Cell Physiology, Université de Lille, Department of Life Sciences, Univ. Lille, Inserm, U1003—PHYCEL, Lille, France, [3] Univ. Lille, CNRS, INSERM, CHU Lille, Centre Oscar Lambret, UMR 9020-UMR 1277-Canther-Cancer Heterogeneity, Plasticity and Resistance to Therapies, Lille, France, [4] Institut Universitaire de France (IUF), Paris, France

Transient Receptor Potential (TRP) cations channels, as key regulators of intracellular calcium homeostasis, play a central role in the essential hallmarks of cancer. Among the multiple pathways in which TRPs may be involved, here we focus our attention on the ones involving small guanosine triphosphatases (GTPases), summarizing the main processes associated with the metastatic cascade, such as migration, invasion and tumor vascularization. In the last decade, several studies have highlighted a bidirectional interplay between TRPs and small GTPases in cancer progression: TRP channels may affect small GTPases activity *via* both Ca^{2+}-dependent or Ca^{2+}-independent pathways, and, conversely, some small GTPases may affect TRP channels activity through the regulation of their intracellular trafficking to the plasma membrane or acting directly on channel gating. In particular, we will describe the interplay between TRPC1, TRPC5, TRPC6, TRPM4, TRPM7 or TRPV4, and Rho-like GTPases in regulating cell migration, the cooperation of TRPM2 and TRPV2 with Rho GTPases in increasing cell invasiveness and finally, the crosstalk between TRPC1, TRPC6, TRPM8, TRPV4 and both Rho- and Ras-like GTPases in inducing aberrant tumor vascularization.

Keywords: transient receptor potential channels, small guanosine triphosphatases, cancer hallmarks, metastasis, molecular signaling, migration, invasion, tumor vascularization

OPEN ACCESS

Edited by:
Sébastien Roger, Université de Tours, France

Reviewed by:
Isaac Jardin, University of Extremadura, Spain
Osama F. Harraz, University of Vermont, United States

***Correspondence:**
Dimitra Gkika
dimitra.gkika@univ-lille.fr

Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *08 July 2020*
Accepted: *11 September 2020*
Published: *29 September 2020*

Citation:
Chinigò G, Fiorio Pla A and Gkika D (2020) TRP Channels and Small GTPases Interplay in the Main Hallmarks of Metastatic Cancer. Front. Pharmacol. 11:581455. doi: 10.3389/fphar.2020.581455

INTRODUCTION

Ca^{2+} signaling plays a central role in the regulation of many important cellular functions and indeed, not surprisingly, a dysregulation of Ca^{2+} homeostasis has been observed in various pathological conditions, including tumorigenesis (Monteith et al., 2017). Changes in Ca^{2+}

Abbreviations: EC, endothelial cell; ECM, extracellular matrix; EGF, epidermal growth factor; EMT, epithelial to mesenchymal transition; ER, endoplasmatic reticulum; FA, focal adhesion; FAK, focal adhesion kinase; GAP, GTPases activating protein; GEF, guanine exchange factor; GDI, guanine-nucleotide dissociation inhibitors; GDP, guanosine diphosphate; GTP, guanosine triphosphate; GTPase, guanosine triphosphatases; MLC, myosin light chain; MMP, membrane-matrix metalloproteinases; PCa, prostate cancer; PI3K, phosphoinositide 3-kinase; PFGF, platelet-derived growth factor; TEC, tumor endothelial cell; TRP, transient receptor potential; VEGF, vascular endothelial growth factor.

signaling leading to dysfunctions in cancer cells are due to alterations in the so-called "Ca^{2+}-signaling toolkit" (Berridge et al., 2003) which includes, among others, ion channels, responsible for altered fluxes within the cell, and several Ca^{2+}-dependent effectors, which mediate various signal transduction pathways in response to altered Ca^{2+} homeostasis.

In recent years, the involvement of Ca^{2+}-permeable channels in neoplastic diseases has been extensively investigated and a direct correlation between their dysregulation and cancer development has been shown (Monteith et al., 2017). Among them, Transient Receptor Potential (TRP) channels have revealed an important involvement in the regulation of many signaling pathways associated with tumor progression (Yang and Kim, 2020). One of the key features of TRP channels is their polymodal activation mechanism and their involvement in different signal transduction pathways. Among the multiple pathways TRPs are signaling through, the ones involving specific intracellular messengers belonging to the family of small guanosine triphosphatases (GTPases) are emerging as essential in tumorigenesis in the last decade. Therefore, in this review, we will focus on the interplay between TRPs and small GTPases, and its role in several aspects of the metastatic process (Aspenström, 2004; Clayton and Ridley, 2020).

The metastatic cascade leads to spread of malignant cells from the primary tumor through the lymph or blood circulation to establish secondary growth in a distant organ; it is a multistep process involving highly complex structural and functional alterations within cancer cells. Primary tumor cells are primed for dissemination by the process of epithelial-mesenchymal transition (EMT), during which they assume a more aggressive mesenchymal-like phenotype. This phenotypic switch allows them to detach from the primary tumor mass and to acquire migratory and invasive properties, in order to move from their original location, migrate and invade the extracellular matrix (ECM) and endothelium to spread to secondary sites and form metastases (Roche, 2018). All the steps involved in metastatic cascade (EMT, cell migration, invasion and tumor vascularization) are regulated by the intracellular Ca^{2+} concentration (Berridge et al., 2003) and specifically by the TRP-mediated calcium influx (Fels et al., 2018), as well as by the most important small GTPases (Rho-like and Ras-like) involved in cytoskeletal dynamics and cell polarity (Ungefroren et al., 2018). Here, we discuss TRPs and small GTPases contribution in cancer progression, focusing on signaling pathways involving a direct interplay between TRPs and small GTPases in three main metastatic cancer hallmarks: migration, invasion and tumor vascularization.

TRP Channels

TRP channels mainly act as signal transducers by altering membrane potential or intracellular Ca^{2+} concentration in response to various environmental stimuli, including physical-chemical stimuli, such as temperature, pH changes, osmolarity, and pressure as well as endogenous and exogenous ligands (Nilius and Owsianik, 2011). TRP channels have been shown to play a central role in carcinogenesis as well as in various late stages of tumor progression. In particular, it has been shown that changes in the expression of TRP channels are correlated with the progression of different types of cancer. To date, most changes involving TRP proteins do not involve mutations in the TRP gene but rather dysregulation of the wild-type TRP protein expression levels, depending on the stage of cancer (Gkika and Prevarskaya, 2011; Lehen'kyi and Prevarskaya, 2011; Bernardini et al., 2015; Shapovalov et al., 2016). Moreover, several recent studies have highlighted a direct correlation between cancer patient survival and TRP channel expression. Tumor differential expression of the main TRP channels discussed in this review and its correlation with patients' survival are summarized in **Table 1**.

These observations strongly indicate that TRPs play a significant role in cancer progression and more specifically in many processes underlying the metastatic cascade, making them promising candidates as both molecular biomarkers and therapeutic targets in various types of cancer (Gkika and Prevarskaya, 2011; Fiorio Pla and Gkika, 2013; Bernardini et al., 2015; Lastraioli et al., 2015; Litan and Langhans, 2015; Fels et al., 2018). It has been shown that TRP-mediated effects on metastatic cancer cell behavior are mainly associated to their Ca^{2+} permeability. Indeed, through the regulation of intracellular Ca^{2+} concentration, both in the cytosol and within subcellular organelles, TRP channels play a key role in many Ca^{2+}-dependent signaling pathways, including those associated with the metastatic cascade such as EMT, migration, invasion and tumor vascularization (Iamshanova et al., 2017). In response to different environmental challenges during the metastatic cascade, like hypoxic, acidic and mechanical cues, cancer cells "re-program" TRPs expression and "misuse" their functions in order to put in place and sustain a more aggressive, metastatic phenotype. However, although most of the TRP-mediated pathways involved in cancer progression are due to alteration of Ca^{2+} homeostasis, it has been also demonstrated an involvement of these channels independent from their Ca^{2+} permeability. As an example, TRPM7 regulation on cell migration is mainly due to Mg^{2+} influx through the channel (Abed and Moreau, 2009; Su et al., 2011). Similarly, the Na^{+}-selective TRPM4 channel has been implicated in cancer migration, although it is inherently Ca^{2+}-impermeable (Vennekens and Nilius, 2007; Cáceres et al., 2015; Holzmann et al., 2015). On the other hand, TRPs' involvement in the metastatic cascade may also be pore-independent, extending the interest in TRPs beyond the field of ion channels (Vrenken et al., 2015). Indeed, TRPM7 promotes many of its biological effects through its peculiar intrinsic kinase activity (Desai et al., 2012; Faouzi et al., 2017; Cai et al., 2018). Moreover, we recently demonstrated the role of TRPM8 in inhibiting vascular endothelial cell migration, which is independent from the pore function of the channel (Genova et al., 2017).

It is therefore evident that, despite the countless advances made in recent years in the study of TRP channels, there are still many aspects to be explored in order to better characterize the main TRP-mediated pathways involved in tumor progression and thus be able to develop new cancer therapies that use TRPs as therapeutic targets.

Small GTPases

The family of small GTPases is composed of a large group of structurally and functionally related proteins, subgrouped into

TABLE 1 | TRPs expression in cancer and correlation with patient survival prognosis.

Channel	Cancer Type	Expression			Prognosis	References
		Healthy/ Benign	Tumor	Invasive		
TRPC1	Breast basal/lymph nodes	Yes	↑	↑	poor	Azimi et al., 2017;
	Breast ductal adenocarcinoma	yes	↑			Dhennin-duthille et al., 2011
TRPC5	Colon	yes	↑	↑	poor	Chen et al., 2017a
TRPC6	Prostate	yes	↑	↑		Yue et al., 2009
	Glioblastoma	yes	↑	↑		Chigurupati et al., 2010
	Esophageal squamous cell carcinoma	yes	↑	↑	poor	Zhang et al., 2013
	Breast/Invasive ductal adenocarcinoma	yes	↑			Guilbert et al., 2008; Dhennin-duthille et al., 2011
TRPM2	Breast	yes	↑	↑	Depends on subtype	Sumoza-Toledo et al., 2016
TRPM4	Prostate	yes	↑			Ashida et al., 2004; Singh et al., 2006
	Cervical	yes	↑			Narayan et al., 2007
TRPM7	Breast	yes	↑	↑	poor	Middelbeek et al., 2012; Meng et al., 2013
	Invasive ductal adenocarcinoma/lymph nodes	yes	↑	↑		Dhennin-duthille et al., 2011; Guilbert et al., 2013
	Pancreatic ductal adenocarcinoma/lymph nodes	yes	↑	↑	poor	Rybarczyk et al., 2012; Yee et al., 2015
	Nasopharyngeal	no	↑	↑	poor	Chen et al., 2015
	Ovarian; Prostate; Melanoma; Sarcoma			↑		Meng et al., 2013
TRPM8	Breast ductal adenocarcinoma	yes	↑	↑		Dhennin-duthille et al., 2011
	Prostate	yes	↑	loss		Tsavaler et al., 2001; Fuessel et al., 2003; Bidaux et al., 2005
	Urothelial carcinoma of bladder	yes	↑	↑	poor	Xiao et al., 2014
	Lung	Very low level	↑			Tsavaler et al., 2001
	Colon	no	↑			Tsavaler et al., 2001
	Melanoma	no	↑			Tsavaler et al., 2001
	Osteosarcoma	yes	↑	↑	poor	Zhao and Xu, 2016
TRPV2	Prostate	no		↑		Monet et al., 2010
	Bladder	yes (full isoform) (short isoform)	↑ ↓	↑ Loss		Caprodossi et al., 2008
	Esophageal squamous cell carcinoma	yes	↑	↑	poor	Zhou et al., 2014
	Gastric	yes	↑	↑	poor	Zoppoli et al., 2019
	Breast (Triple negative/basal subtype)	yes	↑		better	Elbaz et al., 2018
TRPV4	Breast	yes	↑	↑	poor	Lee et al., 2016; Lee et al., 2017
	Gastric		↑		poor	Lee et al., 2016
	Ovarian		↑		poor	Lee et al., 2016
	Glioma	yes	↑	↑	poor	Ou-yang et al., 2018

↑: *increment;* ↓: *reduction.*

six families: Ras, Rho, Arf, Rab, Ran, and RGK. Among them, Ras-like and Rho-like GTPases are the most well characterized. Mechanistically, small GTPases are molecular switches that cycle between an active GTP- bound form and an inactive GDP-bound form. More specifically, these enzymes, once bound to GTP, can catalyse its hydrolysis to GDP and this reaction then results in a conformational change which causes the inactivation of the proteins (Vetter and Wittinghofer, 2001; Cherfils and Zeghouf, 2013). The cycling between GTP- and GDP-bound states is tightly regulated by specific GTPases activating protein (GAPs), which act as negative regulators, promoting the GDP-bound state by increasing the hydrolysis activity of small GTPases, and by guanine exchange factors (GEFs), which act as positive regulators, guiding the replacement of the hydrolysed GDP for a GTP, thus promoting the enzyme active state (Mishra and Lambright, 2016). Moreover, a third family of regulatory proteins called guanine-nucleotide dissociation inhibitors (GDIs) inhibits small GTPases activity by controlling their intracellular localization: GDIs bind to GTPases in their inactive GDP-bound state and sequester them in the cytosol, thus preventing their translocation to intracellular membranes, where activation occurs (Mishra and Lambright, 2016). Indeed, it has been shown that spatial and temporal distributions of the different small GTPases, as well as of their regulators, are important determinants in signaling by small GTPases, determining many aspects of cell behavior (Yarwood et al., 2006; Cherfils and Zeghouf, 2013; Moissoglu and Schwartz, 2014).

The conformational changes following the binding to GTP allow the association of small GTPases with a large number of potential effector proteins such as enzymes and scaffold proteins, which mediate the specific biological effects of each GTPases. Thanks to their ability to interact with a wide number of downstream targets and to co-ordinately activate several molecular processes required for a particular cellular response, small GTPases function as signaling switches in numerous cellular processes. In general, it

has been found that Ras-like GTPases are mainly implicated in regulating cell cycle, differentiation and proliferation, (Shields et al., 2000), whereas Rho-like GTPases are mainly involved in cell morphology, cytoskeletal dynamics and cell polarity (Ridley, 2001). Moreover, Rab GTPases play a key role in many cellular functions, by controlling intracellular trafficking between organelles through vesiculotubular carriers and thus ensuring the spatiotemporal regulation of vesicle traffic (Stenmark, 2009). Considering the central role of Ca^{2+} signaling in many of these processes, it is not surprising that small GTPases activity was found to be strongly related to Ca^{2+} homeostasis (Aspenström, 2004). More specifically, their activation/inactivation may occur through Ca^{2+}-dependent mechanisms acting on specific GEF/GAP proteins or directly on them. Moreover, some GTPases have revealed a direct influence on calcium signaling by regulating the activity of certain calcium channels, including TRPs, by itself or through their effectors (Koopman et al., 2003; Aspenström, 2004; Iftinca et al., 2007; Correll et al., 2008). Finally, several small GTPases collaborate with calcium signaling through the activation of specific Ca^{2+}-related effectors involved in cellular processes, such as cell adhesion, cell migration and exocytosis (Cullen and Lockyer, 2002; Aspenström, 2004; Bader et al., 2004).

Small GTPases expression results dysregulated in several tumors and has been correlated with the progression of the disease (Sahai and Marshall, 2002; Svensmark and Brakebusch, 2019). Without a doubt, Ras is still the most studied small GTPase in cancer, due to the role played by mutated ras genes in the pathogenesis of human tumors (Bos, 1989; Hobbs et al., 2016; Li et al., 2018). Indeed, oncogenic mutations on H-ras, K-Ras, and N-Ras genes have been detected in several tumor types, although the incidence varies greatly (Bos, 1989; Prior et al., 2012; Li et al., 2018). Moreover, some members of the Ras superfamily like Rap1, resulted involved in cancer hallmarks such as migration and angiogenesis, due to their key role in integrin-mediated "inside-out" signaling events (Chrzanowska-wodnicka et al., 2008; Boettner and Van Aelst, 2009; Carmona et al., 2009). As regards the Rho superfamily, its role in many aspects of the metastatic cascade, including EMT, cell migration, invasion and angiogenesis, has been well established (Bryan and D'Amore, 2007; Ungefroren et al., 2018; Clayton and Ridley, 2020).

TRPs-Small GTPases Interplay in Metastatic Cancer

Migration

One of the key steps in the metastatic cascade involves the acquisition of motility by cancer cells. This results from a complex coordination between cytoskeleton dynamics, cellular contractility and cell adhesion rearrangements. Cell migration is a dynamic process characterized by the cycling of four principal steps: after an initial phase in which cell spreading increases about twice with the generation of protrusions at the leading edge and cell adhesion to the ECM increases, then spreading is reduced and thanks to the generation of traction forces cell compacts and detaches at the trailing edge, allowing for cellular movement (Haws et al., 2016). Due to their role as key regulators of cytoskeletal dynamics and cell polarity, it is not surprising that Rho GTPases play a central role in controlling cell migration (Clayton and Ridley, 2020). This process is triggered by cell acquisition of a front-rear end polarity due to the formation of protrusive structures, called filopodia and lamellipodia, at the front edge, opposite to a retracting trailing edge (Llense and Etienne-Manneville, 2015). Filopodia consist of actin filaments organized as long parallel bundles and their formation is dependent on Rho GTPase Cdc42 activity, whereas lamellipodia result from the subsequent Rho GTPase Rac1-mediated branching of actin filaments. The extension of actin-based protrusions is accompanied by the formation at the leading edge of new adhesions that link integrins on the plasma membrane to the F-actin cytoskeleton through talin, vinculin, and focal adhesion kinases (FAKs) and which can mature into focal adhesions (FAs) in a process Rho GTPase RhoA- dependent. Contemporary, RhoA also mediates cytoskeletal rearrangements that lead to the formation of stress fibers, structures composed of bundles of actin and myosin II that have a high contractile capability. The force necessary to pull the cell body forward is engendered by the association of mature FAs with the end of stress fibers and by the actin-myosin cytoskeleton contraction. Finally, the disassembly of FAs in the rear of the migrating cells supports cell retraction at the trailing edge, allowing for cell detachment and movement.

A direct involvement of TRP channels activity in all processes associated with cell migration, including cytoskeletal rearrangement, FA turnover and cellular contractility, has been well established (Fiorio Pla and Gkika, 2013; Canales et al., 2019). Therefore, it is not surprising that several studies have highlighted a tight interplay between TRP channels and Rho GTPases in controlling cell migration during cancer progression. As depicted in **Figure 1**, this interplay is characterized by a bidirectional communication, in which TRP channels promote actin cytoskeleton reorganization through a cation-dependent activation of Rho GTPases, and, conversely, some small GTPases cause changes in TRP channel location, protein–protein interactions and channel gating, thereby modulating their function. More specifically, as described in the following paragraphs, TRPC5, TRPC6, TRPC1, TRPM7, TRPM4, and TRPV4 have been found to affect cell migration through a direct interaction with some small GTPases belonging to the Rho family.

Indeed, TRPC5 and TRPC6 channels have been identified as antagonist regulators of actin remodelling and cell motility in fibroblast and kidney podocytes, mediating the activation of Rac1 and RhoA, respectively (Tian et al., 2010). It has been shown that TRPC5 and TRPC6 trigger antagonistic and mutually inhibitory pathways associated with the maintenance of the balance between contractility and motility. In particular, TRPC5-mediated Ca^{2+} influx induces Rac1 activation, thereby enhancing motility and cell migration. Conversely, TRPC6-mediated Ca^{2+} influx stimulates an increase in RhoA activation, thereby promoting stress fibers formation, cell contractility and the subsequent inhibition of cell migration (Tian et al., 2010) or the disruption of podocytes architecture in glomerular renal diseases, which may lead to proteinuria (Jiang et al., 2011). Interestingly, the functional coupling

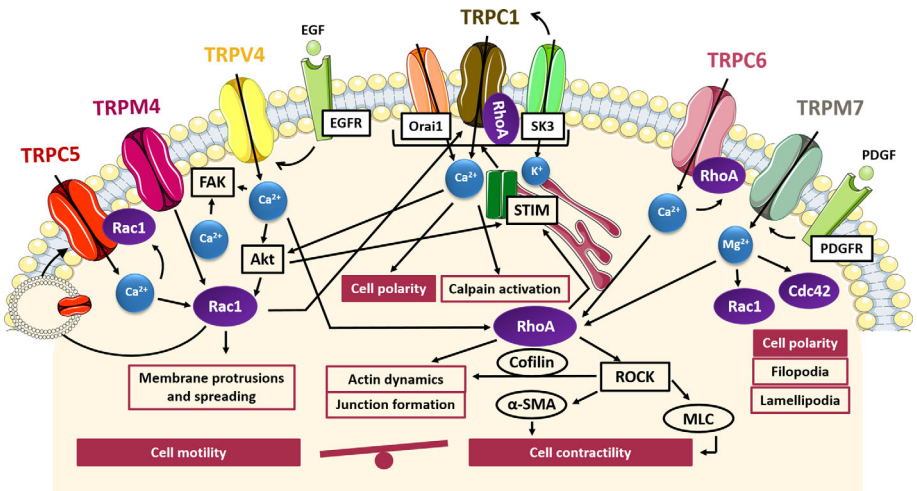


FIGURE 1 | TRP- small GTPases signaling pathways interplay in cell migration. Cartoon depicting TRP channels signaling pathways affecting cell motility and contractility through GTPases. TRPC5, TRPM4 and TRPV4 induce the formation of protrusions and spreading *via* Rac1 activation in a Ca^{2+}-dependent manner and at the same time Rac1 promote the translocation of TRPC5 into the plasma membrane; Rac1 and RhoA through SOCE activation induce TRPC1-mediated cell polarization for directional cell migration; TRPM7 control polarized cell movement through the regulation of Rac1 and Cdc42 in a Mg^{2+}-dependent way; TRPM7, TRPV4, and TRPC6 contribute to actin dynamics and cell contractility through the Mg^{2+}- or Ca^{2+}-mediated activation of RhoA/ROCK pathways. FAK, focal adhesion kinase; Akt, protein-kinase B; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; Orai1, calcium release-activated calcium channel; SK3, small conductance calcium-activated potassium channel 3; STIM, stromal interaction molecule 1; ROCK, Rho-associated protein kinase; α-SMA, alfa-smooth muscle actin; MLC, myosin light chain.

between TRPC5 and TRPC6 with Rac1 and RhoA respectively, gives rise to two distinct molecular complexes predominantly localized to discrete membrane compartments, as detected by co-immunoprecipitation and immunofluorescence (Tian et al., 2010). Of note, the presence of constitutively active Rac1 has been shown to affect TRPC5 channel localization, leading to an increase in plasma membrane abundance of TRPC5 with respect to TRPC6, which, conversely, is predominant in cells expressing constitutively active RhoA. In this regard, RhoA seemed to have no effect on the dynamics of TRPC6 insertion into the membrane, whereas Rac1 was found to promote translocation of TRPC5 into the plasma membrane, according to other findings (Bezzerides et al., 2004). This close interdependence between TRPC5 and Rac1 could suggest a positive feedback mechanism in which the Rac1-mediated TRPC5 insertion from a vesicular pool into the cell membrane leads to enhanced TRPC5-mediated Ca^{2+} influx, which in turn triggers the activation of Rac1 and the subsequent migratory phenotype. Although a direct correlation between TRPC5/TRPC6 and Rac1/RhoA has not yet been established in cancer models, it is possible to speculate that a similar interplay may also occur in the regulation of cancer cells migration, taking into account the evidence for direct involvement of TRPC5 and TRPC6 in the increased migratory potential of several cell types (Greka et al., 2003; Xu et al., 2006; Rampino et al., 2007), including some tumors such as colon cancer and glioblastoma (Chigurupati et al., 2010; Chen L. et al. 2017; Chen Z. et al., 2017). However, whether these effects on tumor migration are dependent on the impact of TRPC5 and TRPC6 activity on cell contractility and motility remains to be clarified.

Similarly, TRPC1 has shown a correlation with an increased migratory phenotype in some tumors, such as glioblastoma, osteosarcoma, thyroid, pancreatic and colon carcinoma (Dong et al., 2010; Asghar et al., 2015; Huang et al., 2015; Guéguinou et al., 2016; Lepannetier et al., 2016). One of the key steps in cell migration is the establishment of a functional and morphological polarity along the axis of movement. In this context, it has been demonstrated that TRPC1 is localized to lipid raft domains at the leading edge of migrating cells and plays a role in determining their polarity and directionality (Fabian et al., 2008; Bomben et al., 2011; Huang et al., 2015). For instance, it has been shown that silencing of TRPC1 in transformed renal epithelial Madin–Darby Canine Kidney-Focus (MDCK-F) cells leads to a decrease in migration associated with a failure of cell polarization and an impaired lamellipodia formation. This effect is due to the partial loss of the local Ca^{2+} gradient at the front edge, needed to establish and maintain the axis of movement in migrating cells (Fabian et al., 2008). The same results were also shown in U2OS bone osteosarcoma cells, in which TRPC1 inhibition or knockdown correlates with a decrease in the percentage of polarized cells and the consequent reduction in cell migration (Huang et al., 2015). However, the link between TRPC1-mediated Ca^{2+} gradients and actin dynamics, as well as the possible involvement of TRPC1-mediated Rho GTPases activation in these processes has not been yet fully characterized. Nonetheless, a direct interaction between TRPC1 and RhoA has been characterized in intestinal epithelial cells (IECs) and in pulmonary arterial endothelial cells (Mehta et al., 2003; Chung et al., 2015). Indeed, TRPC1-RhoA interaction

regulates TRPC1-mediated Ca^{2+} influx through SOCE, stimulating IECs migration after wounding (Chung et al., 2015). A reduction of RhoA/TRPC1 complexes, induced by downregulation or inactivation of either small GTPase or TRP channel, is associated with an inhibition of Ca^{2+} influx after store depletion and the decrease in wound healing after injury (Chung et al., 2015). Besides RhoA, a mechanism involving TRPC1 and Rac1 in promoting colon cancer cell migration has recently been proposed (Guéguinou et al., 2016). Indeed, in HCT-116 colon cancer cells TRPC1 and Rac1 are involved in a complex positive feedback loop in which EGF-induced SOCE activates both Rac1 and STIM1 through Akt pathway; in turn, STIM1 activation promotes translocation of TRPC1 and Orai1 into lipid rafts where SK3 is located and thereby triggers SOCE mediated by the complex SK3/TRPC1/Orai1. At the same time, Akt-mediated Rac1 activation enhances SOCE and thereby SOCE-dependent cell migration through Akt pathway, with subsequent lamellipodia formation and calpain activation. Taken together these data suggest a direct interplay between TRPC1 and Rho GTPases in controlling cell polarity and actin rearrangements during cancer cell migration.

TRPM7 is involved in directional migration in different cell types including migrating fibroblasts, osteoblasts, astrocytes and endothelial cells (ECs) (Abed and Moreau, 2009; Wei et al., 2009; Baldoli et al., 2013; Zeng et al., 2015). Similarly to TRPC1, TRPM7 has been shown to be involved in Ca^{2+} gradient formation, contributing to cell polarization and directional migration (Wei et al., 2009). In particular, TRPM7 is positively correlated with platelet-derived growth factor (PDGF)-induced Mg^{2+} influx as well as high-calcium microdomains "Ca^{2+} flickers", most active at the leading lamella of migrating cells (Abed and Moreau, 2009; Wei et al., 2009). Interestingly, several data have shown that the effects of TRPM7 in controlling cytoskeleton and polarized cell movement are independent of its kinase activity and are associated with its channel function. This has been clearly demonstrated in fibroblasts and neuroblastoma cells where re-expression of TRPM7, as well as a kinase-inactive mutant of TRPM7 on knock out cells, reverted phenotypic changes in cell polarization enhancing cell spreading and adhesion (Clark et al., 2008; Su et al., 2011). In particular, it has been shown that Mg^{2+} plays a central role in TRPM7-mediated control of directional migration in fibroblasts and osteoblasts (Abed and Moreau, 2009; Su et al., 2011). The effects observed by TRPM7 depletion in cell morphology, disruption of actin filaments and myosin fibers and a decrease in the number of FAs, correlates with decreased activity of RhoA GTPase, suggesting a role for TRPM7 in RhoA regulation (Su et al., 2011). Besides its interaction with RhoA, TRPM7 is also functionally coupled with Rac1 and Cdc42: indeed, TRPM7 knockdown prevents Rac1 and Cdc42 activation, with a subsequent deficiency in their ability to form lamellipodia and impaired polarized cell movements (Su et al., 2011). Although Rho GTPases, differing from Ras and Rab proteins, do not require Mg^{2+} for high-affinity nucleotide binding, it has been shown that Mg^{2+} plays a role in regulating nucleotide binding and hydrolysis kinetics in the GEF- and GAP-catalyzed reactions of Rho family GTPases (Zhang et al., 2000). In particular, RhoGAPs exploit Mg^{2+} to achieve high catalytic efficiency and specificity and, conversely, RhoGEFs are negatively regulated by free Mg^{2+}, since the presence of Mg^{2+} significantly decreases the intrinsic dissociation rates of the nucleotides. This finding may suggest that one role of GEFs is to displace bound Mg^{2+} from Rho proteins in order to efficiently perform their function and dissociate nucleotide from Rho GTPases (Zhang et al., 2000). Taken together this data revealed an interesting and so far little investigated interplay between TRP channel and Rho GTPases in controlling cell migration mediated by Mg^{2+} homeostasis.

More recently, another member of the TRPM subfamily, TRPM4, has been recognized as the first TRP channel to be part of the adhesome that is the set of protein components of FAs required for migration and contractility (Cáceres et al., 2015). Indeed, it has been shown that TRPM4 localises to FAs in different cell types and that its suppression impaires FAs relocation and lamellipodia formation, leading to a reduced cellular spreading and migration in mouse embryonic fibroblasts. Actually, FA turnover plays a key role in cell migration, contributing to the generation of the traction forces necessary for cellular movements. TRPM4-mediated effects on cell migration are at least partially due to the activation of Rac1 GTPase. Indeed, it has been observed that upon silencing TRPM4 the serum-induced activation of Rac1 and lamellipodial distribution are significantly reduced, suggesting a direct cooperation between TRPM4 and Rac1 in the regulation of cellular spreading. Moreover, it has been demonstrated that TRPM4 pharmacological inhibition caused retarded skin wound healing *in vivo*, affecting cell contractility (Cáceres et al., 2015). TRPM4 has been found to affect the migratory behaviour of many cell types, including prostate cancer cells (Holzmann et al., 2015). Although TRPM4 itself is Ca^{2+}-impermeable, its contribution to cell migration through the regulation of intracellular Ca^{2+} signaling has been well established (Vennekens and Nilius, 2007). For instance, in androgen-insensitive prostate cancer cells, it has been shown that TRPM4 acts as an important negative feedback regulator of SOCE, thus promoting cell migration (Holzmann et al., 2015). However, further investigations are needed to deepen the knowledge of the molecular mechanism underlying the pro-migratory effect of TRPM4 on prostate cancer cell migration.

To date, several experimental pieces of evidence have revealed a critical role of TRPV4 in regulating the migratory properties of many tumors, including liver, breast and gastric cancer (Vriens et al., 2004; Lee et al., 2016; Xie et al., 2017). However, little is known about the molecular mechanism driving this process. Nonetheless, it has been shown that TRPV4 is involved in the dynamics of trailing adhesions, likely through an interplay with other cation channels or proteins present at the FA sites (Mrkonjić et al., 2015). A direct correlation between TRPV4 and RhoA/ROCK pathways has been revealed in cardiac fibroblast remodelling and myofibroblast contraction. In particular, it has been shown that after stimulation with growth factors, TRPV4 contributes to cell contractility by increasing the actin protein α-SMA expression and incorporation

into stress fibers, through the Ca^{2+}-mediated activation of RhoA/ROCK pathways (Tomasek et al., 2006; Adapala et al., 2013; Thodeti et al., 2013). Another study reported a role of TRPV4 in the modulation of adherent-junctions, mediated by the TRPV4-dependent activation of Rho GTPases, thereby promoting actin fibers organization and junctions formation (Sokabe and Tominaga, 2010). Furthermore, the exogenous up-regulation of TRPV4 in breast cancer has been shown to aid actin dynamics and lead to higher activation of cofilin, a downstream protein effector of RhoA/ROCK pathways that promotes actin filaments depolymerisation, thus conferring cellular "softness" and promoting transendothelial migration (Lee et al., 2016). Accordingly, TRPV4 knockdown reduced migration, invasion and transendothelial migration in breast cancer cells (Lee et al., 2016). Finally, a recent study of Ou-yang and co-worker (Ou-yang et al., 2018) has described the Akt/Rac1 signaling pathway through which TRPV4 promotes migration and invasion in glioma cancer cells (Ou-yang et al., 2018). Mechanistically, agonist-mediated TRPV4 activation promoted the activation of Rac1 by targeting Akt for phosphorylation, thus enhancing glioma cell migration and invasion (Ou-yang et al., 2018). Accordingly, in gastric cancer, TRPV4-mediated Ca^{2+} influx promotes cell migration through the activation of the downstream Akt/β-catenin pathways (Xie et al., 2017). Collectively, these data support a direct interplay between TRPV4 and small GTPases in controlling cytoskeletal remodeling aimed to confer migratory phenotypes.

Invasion

In order to reach lymph- and bloodstreams and to colonize sites distant from the primary tumor, cancer cells have to acquire, beyond migratory phenotype, the ability to degrade ECM. Consequently, invasion is another key step of the metastatic cascade. Invasiveness of cancer cells comes from their ability to produce special protrusions called invadopodia, which are actin-rich protrusions of the plasma membrane with proteolytic activity. Once matured, invadopodia recruit proteolytic enzymes such as membrane-matrix metalloproteinases (MMPs), which are endopeptidases able to locally degrade ECM, allowing cell invasion. Among them, MMP-2 and MMP-9 are considered the most important in metastasis, since they were often aberrantly expressed in tumors (Jabłońska-trypuć et al., 2016). Both TRP channels and small GTPases have been implicated in increased tumor invasiveness through the induction of MMPs expression (Betson and Braga, 2003; Yang and Kim, 2020). Indeed, TRPM2, TRPM7, TRPM8, TRPV2, and TRPC1 have shown to cause upregulation of MMP9 in a Ca^{2+}-dependent manner in gastric, bladder, oral squamous, prostate and thyroid cancer cells respectively (Monet et al., 2010; Okamoto et al., 2012; Asghar et al., 2015; Cao et al., 2016; Chen L. et al. 2017; Chen Z. et al., 2017; Almasi et al., 2019). Additionally, TRPM2, TRPM7, and TRPC1 activity have been also correlated with MMP2 production in gastric, lung, pancreatic and thyroid cancer (Asghar et al., 2015; Rybarczyk et al., 2017; Liu et al., 2018; Almasi et al., 2019). On the other hand, Rho and Rac GTPases activation has been correlated with increased MMPs expression in different cancer cell types (Zhuge and Xu, 2001; Abécassis et al., 2003; Santibáñez et al., 2010; Jacob et al., 2013).

TRPV2 and TRPM2 have been shown to affect cell invasiveness through signaling pathways involving small GTPases, which are summarized in **Figure 2**.

In particular, TRPV2 has been found to positively correlate with prostate cancer (PCa) invasiveness, promoting PCa progression to the aggressive castration-resistant stage (Monet et al., 2010). More specifically, it has been shown that siRNA-mediated silencing of TRPV2 leads to a decrease in MMP-2 and MMP-9 expression, reducing growth and invasive properties of PC3 prostate tumors established in nude mice xenografts (Monet et al., 2010). The mechanism by which the Ca^{2+} influx mediated by TRPV2 is linked with the up-regulation of MMPs has not been characterized. However, another study has revealed that TRPV2 trafficking to the plasma membrane correlates with enhanced cell migration and invasion in PCa, *via* the phosphoinositide 3-kinase (PI3K) pathway (Oulidi et al., 2013). Taking into account evidence of a PI3K-mediated activation of Rac1 in several tumor models (Haws et al., 2016; Ungefroren et al., 2018) and considering that Rac1 has been found to regulate TRPV2 intracellular trafficking in fibrosarcoma cells (Nagasawa and Kojima, 2015), it is possible to speculate that in PCa a PI3K-mediated Rac1 activation may allow the translocation to the plasma membrane of the "*de novo*" expressed TRPV2 in PC3 cells, thus giving rise of the TRPV2-mediated increase of cytosolic Ca^{2+} concentration responsible for MMPs overexpression. Confirming this hypothesis, Rac1 has been related to the up-regulation of MMP-2 and MMP-9 in fibrosarcoma (Zhuge and Xu, 2001) and transformed keratinocytes (Santibáñez et al., 2010), respectively. Unlike in PCa, TRPV2 has been found to suppress the invasiveness of fibroblast-like synoviocytes (FLS), which have an aggressive and invasive behaviour resembling that of cancer cells (Laragione et al., 2015). TRPV2 activation has been associated both *in vitro* and *in vivo* with reduced cell invasiveness and a down-regulation of the IL-1β-induced expression of MMP-2 and MMP-3 (Laragione et al., 2015). More recently, the cell signaling events mediating this TRPV2 suppressive activity have been characterized (Laragione et al., 2019). Interestingly, a direct interplay between TRPV2 channel function and RhoA/Rac1 GTPases activity in suppressing FLS cell invasion has been proposed (Laragione et al., 2019). In particular, it has been shown that, upon stimulation with the commercially-available TRPV2-specific agonist O1821, the channel nearly disappears from the plasma membrane, as well as integrins αv, β1, and β3, involved in cell binding to ECM. Concomitantly, a decrease in cell adhesion and the number of thick actin filaments and a reduction in lamellipodia formation are observed. Mechanistically, it has been found that O1821-induced TRPV2 activation causes a decrease in both RhoA and Rac1 activation, giving rise to the observed inhibition of actin filaments and lamellipodia formation, respectively. Moreover, TRPV2 activation significantly increases levels of phosphorylated (inactive) cofilin and affects the localization of active cofilin keeping it in the cytosol away from cell protrusions and lamellipodia, in which normally it exerts its function on actin remodeling, upon activation by Rac1. Considering the active involvement of Rac1 in TRPV2 trafficking found by Nagasawa and Kojima (2015), Rac1

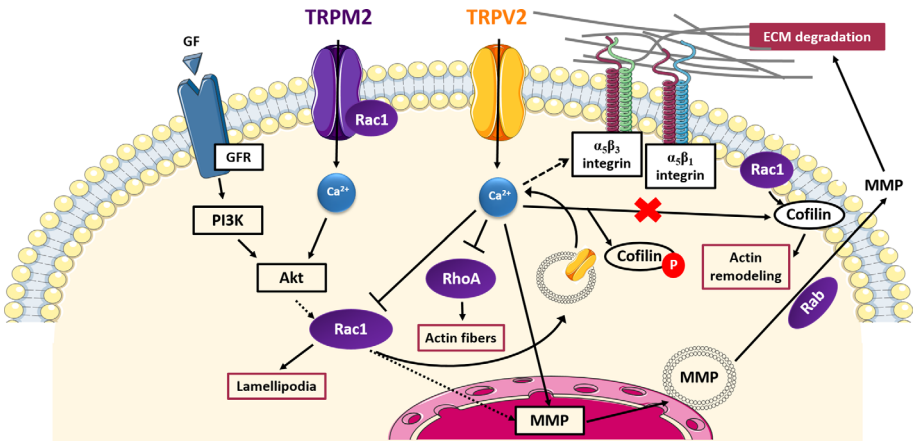


FIGURE 2 | TRP- small GTPases signaling pathways interplay in cell invasion. Cartoon depicting TRP channels signaling pathways affecting cell invasiveness through GTPases. Rac1 promotes the translocation of TRPV2 into the plasma membrane and thus TRPV2-mediated increased in MMPs expression; TRPV2 affects also cell adhesion and invasion interfering with integrin-mediated signalling and inhibiting Rac1, RhoA, and cofilin activation by Rac1 in a Ca^{2+}-dependent manner; TRPM2 and Rac1 physically interact with each other, mutually influencing their activity and lead to an increase in MMPs production; MMPs exocytosis is mediated by the Rab superfamily of small GTPases. GF, growth factor; GFR, growth factor receptor; PI3K, phosphoinositide-3 kinase; Akt, protein-kinase B; MMP, membrane-matrix metalloproteinase; ECM, extracellular matrix.

inhibition through TRPV2 described by Laragione *et al.* might also explain the observed reduction of TRPV2 expression on the plasma membrane after channel stimulation, suggesting an intriguing negative feedback loop between TRPV2 and Rac1 regulation. Indeed, TRPV2 activation on the plasma membrane may inhibit in a Ca^{2+}-dependent manner Rac1 activation, which in turn resulted in a decrease of TRPV2 expression on the cell surface. Taken together these data account for a possible two-sided interplay between Rac1 and TRPV2, based on which TRPV2 may exert both pro- and anti-invasiveness activities depending on cell type. In the first case Rac1 is activated by the PI3K pathway, thus allowing the overexpression of the constitutively active TRPV2 on the plasma membrane and the increased calcium flow responsible for MMPs overexpression and the increased cell invasiveness (Nagasawa and Kojima, 2015); in the second case TRPV2, upon activation by external stimuli, causes Rac1 inactivation and the inhibition of pro-invasive intracellular pathways as well as the expression of TRPV2 in the membrane through a negative feedback mechanism (Laragione et al., 2019). Therefore, in an interesting interchangeable relationship, TRPV2 may act as either modulator or effector of Rac1, depending on cell type, thus reflecting the bivalent activity showed by the channel in cell invasiveness.

TRPM2 offers another potential field of investigation on TRP-small GTPase relationship in cancer cells' ability to invade surrounding tissues. In fact, it has been recently proven that TRPM2 causes an increase in MMP-9 production in gastric cancer (Almasi et al., 2019). As in the case of TRPV4, the function of TRPM2 appears to be regulated by the Akt pathway, known to regulate the activity of several GTPases including Rac1 (Ho et al., 2010), whose interaction with TRPM2 has been already well established in response to oxidative stress (Gao et al., 2014) (**Figure 2**). In this regard, it has been demonstrated that TRPM2 and Rac1 physically interact with each other, mutually influencing their activity, similarly to what was observed between TRPC5 and Rac1 in podocytes previously described (Bezzerides et al., 2004; Tian et al., 2010). Thus, we could speculate a TRPM2-Akt-Rac1 axis in the modulation of MMP-9 expression in gastric cancer.

Tumor Vascularization

Tumor vascularization, is a critical step in the metastatic cascade since the formation of new blood vessels is crucial not only to provide sufficient oxygen and nutrients and thus promoting the continuous growth of tumors but also to drive tumor spread and metastasis. Tumor vascularization, promoted by the tumor cells themselves through the secretion of several growth factors, results aberrant and leads to the formation of new vessels characterized by abnormal morphology, irregular blood flow, and distribution, non-uniform pericyte coverage and hyper-permeability (Carmeliet and Jain, 2000; Carmeliet and Jain, 2011). TRP channels are widely expressed within vascular ECs and several data correlate aberrant TRP channels expression and/or activity with tumor vascularization, thanks to their high sensitivity to both pro-angiogenic signals and subtle changes in the local microenvironment (Brossa et al., 2019; Negri et al., 2020; Scarpellino et al., 2020). TRP channels have been related to critical steps of tumor vascularization, including cell adhesion, cell migration, enhanced permeability and *in vitro* angiogenesis (Fiorio Pla and Gkika, 2013). Similarly, several Rho and Ras small GTPases, thanks to their contribution to actin dynamics, cell contractility and integrin-mediated "inside-out" signaling events, have been found to be dysregulated during tumor vascularization (Carmona et al., 2009; Chrzanowska-Wodnicka, 2010). Some common pathways involving both TRP channels and small GTPases in determining aberrant tumor vascularization have been described, such as those

concerning TRPV4, TRPM8, TRPC1, and TRPC6, depicted in **Figure 3**.

TRPV4 has been the first TRP channel to be clearly implicated in tumor angiogenesis, although it can have both pro-angiogenic and anti-angiogenic effects depending on tumor type. In particular, TRPV4-mediated Ca^{2+} signals are implicated in tumor-derived endothelial cells (TECs) migration *via* a membrane-stretch activated arachidonic acid release and subsequent actin remodeling and TRPV4 insertion in the plasma membrane (Fiorio Pla et al., 2012). These data are in accordance with previous results showing that TRPV4 is required for shear stress EC reorientation in an integrin-dependent manner (Thodeti et al., 2009). However, in different TECs models, TRPV4 results to be down-regulated as compared to normal tissues. Moreover, this down-regulation is correlated with aberrant mechano-sensitivity of TECs towards ECM stiffness and subsequently with enhanced cell motility and abnormal angiogenesis. Subcutaneous injection of Lewis Lung Carcinoma cells in TRPV4 knockout mice leads to increased vascular density, higher vessel diameter, and reduced pericyte coverage, overall resulting in enhanced tumor growth (Adapala et al., 2016). More recently, it has also been shown by the same group that TRPV4 silencing causes a significant decrease in VE-cadherin expression at cell-to-cell junctions, with the subsequent increase in vascular leakage (Cappelli et al., 2019). Accordingly, overexpression or pharmacological stimulation of TRPV4 with GSK1016790A has been shown to lead to a "normalization" of the vascular endothelium, enhancing the permeability of chemotherapeutic drugs, and basically blocking tumor growth (Adapala et al., 2016). In particular, TRPV4-mediated "normalization" of aberrant capillary-like tubules *in vitro* is achieved by restoring the mechano-sensitivity of ECs toward ECM stiffness through the blockade of basal Rho activity (Adapala et al., 2016). In this contest, it was previously shown that the aberrant TECs mechano-sensitivity of TECs in response to ECM stiffness and cyclic strain results, at least partially, due to constitutively high level of Rho/ROCK basal activities (Ghosh et al., 2008). Moreover, ECs isolated from TRPV4 knockout (KO) mice display higher basal Rho activity as compared to EC WT and the inhibition of the Rho/ROCK pathway in TRPV4KO mice results in vessel normalization, confirming the role of TRPV4 as an important modulator of Rho activity also *in vivo* (Thoppil et al., 2016).

In agreement with other recent findings which have highlighted non-conducting functions of TRP channels in many processes, including the regulation of cytoskeletal dynamics (Vrenken et al., 2015), our recent study (Genova et al., 2017) has unveiled that TRPM8 inhibits ECs migration in a Ca^{2+}-independent manner. TRPM8 is mainly localized in the endoplasmatic reticulum (ER) in ECs and its expression results remarkably down-regulated in breast TECs (BTECs) as compared to healthy human microvascular ECs (HMECs) and human umbilical vein ECs (HUVECs) (Genova et al., 2017). Mechanistically, TRPM8 inhibits ECs migration and spheroid sprouting by trapping Rap1 intracellularly, thereby preventing its relocation toward the plasma membrane, which is required to activate β_1-integrin signaling. Inactive Rap1 (GDP- bound) physically interacts with the N-terminal tail of TRPM8 and interestingly, its retention within the cytosol occurs also in the presence of a TRPM8 pore mutant, demonstrating that TRPM8 inhibitory effects on ECs migration and adhesion by trapping Rap1 are independent from its channel function (Genova et al., 2017). Curiously, TRPM8 activation through specific channel agonists (icilin and menthol) or endogenous activators such as

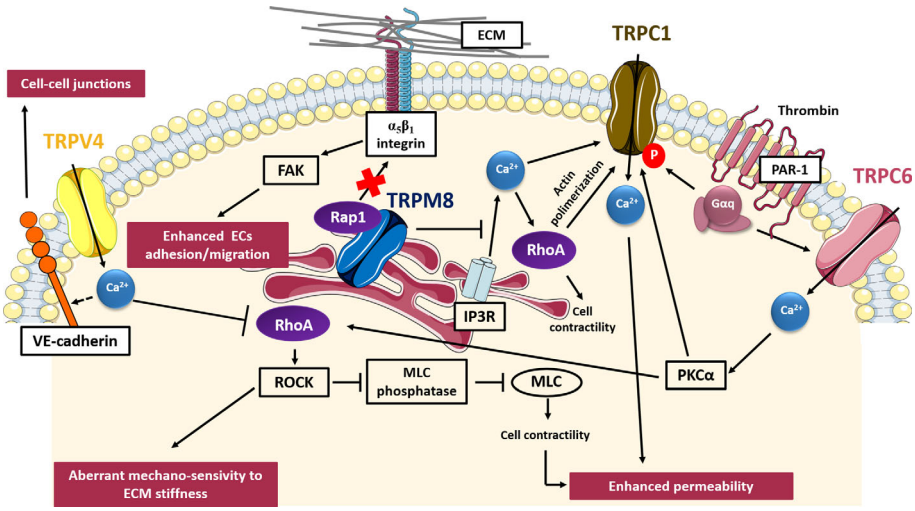


FIGURE 3 | TRP- small GTPases signaling pathways interplay in aberrant tumor vascularization. Cartoon depicting TRP channels signaling pathways affecting tumor vascularization through GTPases. TRPV4 induces aberrant mechano-sensivity to ECM through the Ca^{2+}-dependent inhibition of Rho/ROCK pathway; TRPC1 and TRPC6 enhance vessels permeability affecting cell contractility through a Ca^{2+}-mediated regulation of RhoA; on the contrary, TRPM8 exerts a protective role in tumor vasculature permeability, inhibiting the store-operated RhoA activation and subsequent cell contraction; TRPM8 also inhibits ECs adhesion and migration, impairing activation of β_1-integrin through the intracellular retention of Rap1. VE-cadherin, vascular E-cadherin; FAK, focal adhesion kinase; ROCK, Rho-associated protein kinase; MLC, myosin light chain; PKCα, protein kinase C alpha; IP3R, inositol trisphosphate receptor; PAR-1, protease-activated receptor-1.

prostate-specific antigen (PSA), significantly reduces the amount of active Rap1-GTP bound and enhanced its inhibitory effect on migration, thus raising questions about the possible effects of agonists on TRPM8 besides pore gating. TRPM8-mediated inhibition of Rap1 cytoplasm–plasma membrane trafficking impairs the activation of inside-out signaling pathway known to trigger the conformational activation of β_1-integrin and, consequently, cell adhesion and migration. Indeed, icilin-stimulated TRPM8 significantly inhibits active-β_1 integrins as revealed by immunofluorescence assays. In addition, agonist-induced TRPM8 stimulation leads to a significant decrease in FAK phosphorylation, suggesting the involvement of FAK as a downstream effector of the β_1-integrin pathway affected by TRPM8 (Genova et al., 2017). This study has highlighted how TRP channels may regulate GTPases activity not only by the generation of local Ca^{2+} fluxes, but also acting through physical interactions which affect their intracellular localization and thus their activity. Indeed, as well as TRP channels, also small GTPases are used for spatial and temporal control of cell behavior, as reported by several studies (Yarwood et al., 2006; Wang et al., 2012; Moissoglu and Schwartz, 2014). Another evidence of a TRPM8 interplay with small GTPases in controlling ECs behavior comes from a study of Sun and coworkers (Sun et al., 2014), which has revealed a role of TRPM8 in vasoconstriction and hypertension through attenuating RhoA/Rho kinase pathway. In particular, the authors have shown that TRPM8 activation by menthol attenuates vasoconstriction by inhibiting the RhoA/ROCK pathway in wild-type mice, but not in TRPM8KO mice (Sun et al., 2014). TRPM8 effect is associated with inhibition of intracellular calcium release from the sarcoplasmic reticulum, thus reducing Ca^{2+}-mediated activation of RhoA/ROCK and ECs contraction. Since several experiments have correlated ECs contraction with vessel permeability (Hicks et al., 2010), this study could suggest a protective role of TRPM8 not only in ECs migration, but also in tumor vasculature permeability.

The increased permeability shown by tumor vessels may be induced by several factors, including hypoxia and inflammatory signals, such as thrombin. In this regard, TRPC6 and TRPC1 have been implicated in thrombin-induced hyper-permeabilization of ECs through the RhoA/ROCK signaling pathway. Thrombin is a serine-protease which binds specifically to the protease-activated receptor-1 (PAR-1) on endothelial cell surface, inducing a signaling cascade, which results in an increase in ECs contraction, changes in cell shape and finally, in the development of tiny inter-endothelial junctional gaps that lead to increased endothelial permeability. Interestingly, TRPC6 activation is promoted by thrombin stimulation through the α subunit of G protein-coupled receptors ($G\alpha_q$) (Singh et al., 2007). Then, TRPC6-mediated Ca^{2+} influx leads to the activation of PKCα and thereby induces RhoA activity and ECs contraction, with subsequent cell shape changes, inter-endothelial gaps formation and increase in EC permeability (Singh et al., 2007). Indeed, upon thrombin stimulation it has been shown that RhoA activates its downstream effector ROCK, which in turn promotes myosin light chain (MLC) phosphatase regulatory subunit phosphorylation, reducing its phosphatase activity (Birukova et al., 2004). TRPC6 have also been found to play a central role in determining the angiogenic potential of glioma cells, since its inhibition affected EC tube formation *in vitro* by reducing the number of branch points (Chigurupati et al., 2010). Moreover, TRPC6 has been shown to exert pro-angiogenic effects by affecting vascular endothelial growth factor (VEGF)-induced calcium influx in ECs. Although there are not robust data on GTPases-mediated TRPC6 functions in angiogenesis, recently Zahra and coworkers defined an important role of RhoA in ECs proliferation, migration, invasion and sprouting triggered by important angiogenesis inducers, including VEGF (Zahra et al., 2019), thus suggesting a possible interplay between TRPC6 and RhoA in VEGF-induced angiogenesis.

Similarly to TRPC6, also TRPC1 has been found to have a role in increasing ECs permeability through RhoA activation in response to thrombin stimulation. In this regard, an intriguing mechanism depicting RhoA as a TRPC1 modulator in human pulmonary arterial endothelial cells has been proposed by Mehta and co-worker (Mehta et al., 2003). They showed that RhoA, upon thrombin-induced activation, forms a complex with IP_3R and TRPC1, which then translocates to the plasma membrane, where TRPC1 can mediate a store depletion-induced Ca^{2+} entry and the resultant increase in endothelial permeability. Moreover, it has been demonstrated that RhoA-induced TRPC1-IP_3R association is dependent on actin filament polymerization, since its inhibition hinders both the association and Ca^{2+} entry (Mehta et al., 2003). Moreover, TRPC1 serine/threonine phosphorylation by PKCα is crucial for inducing Ca^{2+} influx and consequently EC permeability (Ahmmed et al., 2004). However, the mechanism by with PKCα integrates with RhoA in ECs to trigger TRPC1-mediated Ca^{2+} influx remained to be characterized. Nonetheless, considering the previously described role of PKCα as a downstream effector of TRPC6 (Singh et al., 2007), it is possible that the PKCα-dependent RhoA-induced TRPC1 activation gives rise to a positive feedback loop that overall leads to a persistent increase endothelial permeability. This study provides an example of pathway in which small GTPases do not act as TRP Ca^{2+}-dependent effectors, but rather as modulators of TRP channel activity, influencing protein-protein interactions and channel gating, and thus corroborating the bidirectional nature of this complex interplay.

Conclusions and Perspectives

TRPs and small GTPases show a direct interplay in cancer progression, characterized by a bidirectional communication, whereby TRP channels have been shown to affect small GTPases activity *via* both Ca^{2+}-dependent or Ca^{2+}–independent pathways, and conversely some small GTPases may affect TRP channels activity through the regulation of their intracellular trafficking to the plasma membrane or acting directly on channel gating (**Table 2**). In most cases, TRP-GTPase interaction is mediated by Ca^{2+} signals, triggered by TRP-mediated Ca^{2+} influx through the plasma membrane induced by growth factors, specific ligands or mechanical stimuli. In some specific cases, such as TRPM7, TRP-mediated small GTPases activation/inhibition may be supported by Mg^{2+} rather than Ca^{2+} homeostasis regulation. Moreover, also Ca^{2+}-impermeable TRP channels like TRPM4 have been found to

TABLE 2 | TRPs-small GTPases relationship in metastatic cancer hallmarks.

Hallmark	TRP Channel		GTPase	Biological Effect	Reference
Migration	TRPC1	Ca^{2+} ←	RhoA	↑	(Mehta et al., 2003; Chung et al., 2015)
		Ca^{2+} ↔	Rac1	↑	(Guéguinou et al., 2016)
	TRPC5	Ca^{2+} ↔	Rac1	↑	(Bezzerides et al., 2004; Tian et al., 2010)
	TRPC6	Ca^{2+} →	RhoA	↓	(Tian et al., 2010)
	TRPM4		Rac1	↑	(Cáceres et al., 2015)
	TRPM7	Mg^{2+} →	RhoA	↑	(Su et al., 2011)
		Mg^{2+} →	Rac1 Cdc42		(Su et al., 2011)
	TRPV4	Ca^{2+} →	RhoA	↑	(Tomasek et al., 2006; Adapala et al., 2013; Thodeti et al., 2013; Lee et al., 2016)
		Ca^{2+} →	Rac1	↑	(Ou-yang et al., 2018)
Invasion	TRPM2	Ca^{2+} ↔	Rac1	↑	(Gao et al., 2014)
	TRPV2	←	Rac1	↑	(Nagasawa and Kojima, 2015)
		Ca^{2+} ┤	RhoA/ Rac1	↓	(Laragione et al., 2019)
Aberrant tumor vascularization	TRPC1	←	RhoA	↑	(Mehta et al., 2003)
	TRPC6	Ca^{2+} →	RhoA	↑	(Singh et al., 2007)
	TRPM8	┤	Rap1	↓	(Genova et al., 2017)
		┤	RhoA	↓	(Sun et al., 2014)
	TRPV4	┤	Rho	↓	(Thoppil et al., 2016; Adapala et al., 2016)

→: TRP enhances GTPase activity; ←: GTPase enhance TRP activity; ↔: bidirectional activation; ┤: TRP inhibits GTPase activity; ↑: increase; ↓: decrease.

regulate small GTPases activity, probably through an indirect control on other Ca^{2+}-signaling pathways. Finally, alternative regulatory pathways that go beyond the canonical ones involving cation homeostasis have been characterized for TRP-mediated small GTPases regulation. For instance, it has been shown that some TRPs, such as TRPM8 in ECs, may act similarly to a GDI-like protein, inhibiting small GTPases activity by physically trapping and restraining them within a specific cellular compartment and thus preventing their switch to the active form that generally occurs at the plasma membrane. Although in most cases small GTPases act as TRP effectors, there are some evidence about the role of small GTPases as modulators of TRP channel activity. Indeed, it has been shown that some small GTPases may affect TRPs channel activity by influencing channel trafficking, gene expression, protein-protein interactions and channel gating. In some cases, positive feedback loop mechanisms, wherein TRP channels activate small GTPases which in turn increase TRPs insertion to the plasma membrane, have also been described. In addition, some studies have highlighted that effector/modulator roles may be interchangeable between TRPs and small GTPases depending on cell type. For instance, it has been shown that Rac1 may act as either modulator or effector of TRPV2 activity, thus determining opposite effects on cell invasion, depending on cell type.

In addition to the examples of a direct TRP-small GTPases interaction in metastatic cancer hallmarks reported in this review, many studies suggest other possible signal transduction pathways associated with tumor progression involving both TRPs and small GTPases. For instance, they have both been implicated in the regulation of MMPs production through pathways like IP3K/Akt and Hsp90α-uPA-MMP2 that offer many points of contact in between them (Koike et al., 2000; Zhuge and Xu, 2001; Yang et al., 2015; Luo et al., 2016; Rybarczyk et al., 2017; Liu et al., 2018). However, to date, a direct correlation between TRPs and small GTPases in these signal transduction pathways has not yet been established but remains to be deeper clarified. Moreover, several studies have reported a role of both these two superfamilies of molecules in promoting another key step of the metastatic cascade that is EMT. In response to the same growth factor/cytokines-induced signaling pathways both TRPs and small GTPases are able to induce the up-regulation of mesenchymal-like markers such as vimentin and the down-regulation of epithelial-like markers such as E-cadherin through the direct regulation of transcriptional factors including STAT3, Snail and Twist (Simon et al., 2000; Davis et al., 2014; Liu et al., 2014; Yang et al., 2015; Chen L. et al., 2017; Kim et al., 2018 Liu et al., 2019; Seiz et al., 2020). However, a synergistic cooperation between TRPs and Rho GTPases during the early stage of growth-factor induced EMT is still only speculation, since to the best knowledge of the authors a direct correlation between TRPs and small GTPases effects on EMT has not yet been characterized. In conclusion, the interplay between

TRP channels and small GTPase in cancer progression is only partially investigated to date and further investigations are required to shed light on many other aspects of this interesting crosstalk in cancer not well known.

AUTHOR CONTRIBUTIONS

DG conceived the study. GC provided wrote and edited the manuscript and designed the figures. AP revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

GC was supported by University of Torino as part of the PhD Program in Complex Systems for Life Sciences. DG is supported by the Institut National du Cancer (INCa- PLBIO14-213) and the Institut Universitaire de France (IUF). GC, AP, and DG were supported by the International Associated Laboratory (LAI) agreement (CaPancInv). AP was supported by grants from the Italian Ministry of Instruction, University and Research (MIUR), PRIN grant "Leveraging basic knowledge of ion channel network in cancer for innovative therapeutic strategies (LIONESS)" (grant number 20174TB8KW).

REFERENCES

Abécassis, I., Olofsson, B., and Schmid, M. (2003). RhoA Induces MMP-9 Expression at CD44 Lamellipodial Focal Complexes and Promotes HMEC-1 Cell Invasion. *Exp. Cell Res.* 291 (2), 363–376. doi: 10.1016/j.yexcr.2003.08.006

Abed, E., and Moreau, R. (2009). Importance of Melastatin-like Transient Receptor Potential 7 and Magnesium in the Stimulation of Osteoblast Proliferation and Migration by Platelet-Derived Growth Factor. *Am. J. Physiol. - Cell Physiol.* 297 (2), 360–368. doi: 10.1152/ajpcell.00614.2008

Adapala, R. K., Thoppil, R. J., Luther, D. J., Paruchuri, S., Meszaros, J. G., Chilian, W. M., et al. (2013). Cellular Cardiology TRPV4 Channels Mediate Cardiac Fi Broblast Differentiation by Integrating Mechanical and Soluble Signals. *J. Mol. Cell. Cardiol.* 54, 45–52. doi: 10.1016/j.yjmcc.2012.10.016

Adapala, R. K., Thoppil, R. J., Ghosh, K., Cappelli, H., Dudley, A. C., Paruchuri, S., et al. (2016). Activation of Mechanosensitive Ion Channel TRPV4 Normalizes Tumor Vasculature and Improves Cancer Therapy. *J. Autism Dev. Disord.* 35 (3), 314–322. doi: 10.1097/CCM.0b013e31823da96d.Hydrogen

Ahmmed, G. U., Mehta, D., Vogel, S., Holinstat, M., Paria, B. C., Tiruppathi, C., et al. (2004). Protein Kinase Cα Phosphorylates the TRPC1 Channel and Regulates Store-Operated Ca 2 Entry in Endothelial Cells *. *J. Biol. Chem.* 279 (20), 20941–20949. doi: 10.1074/jbc.M313975200

Almasi, S., Sterea, A. M., Fernando, W., Clements, D. R., Marcato, P., Hoskin, D. W., et al. (2019). TRPM2 Ion Channel Promotes Gastric Cancer Migration, Invasion and Tumor Growth through the AKT Signaling Pathway. *Sci. Rep.* 9 (1), 4182. doi: 10.1038/s41598-019-40330-1

Asghar, M. Y., Magnusson, M., Kemppainen, K., Sukumaran, P., Löf, C., Pulli, I., et al. (2015). Transient Receptor Potential Canonical 1 (TRPC1) Channels as Regulators of Sphingolipid and VEGF Receptor Expression: Implications for Thyroid Cancer Cell Migration and Proliferation. *J. Biol. Chem.* 290 (26), 16116–16131. doi: 10.1074/jbc.M115.643668

Ashida, S., Nakagawa, H., Katagiri, T., Furihata, M., Iiizumi, M., Anazawa, Y., et al. (2004). Molecular Features of the Transition from Prostatic Intraepithelial Neoplasia (PIN) to Prostate Cancer: Genome-Wide Gene-Expression Profiles of Prostate Cancers and PINs. *Cancer Res.* 64 (17), 5963–5972. doi: 10.1158/0008-5472.CAN-04-0020

Aspenström, P. (2004). Integration of Signalling Pathways Regulated by Small GTPases and Calcium. *Biochim. Biophys. Acta - Mol. Cell Res.* 1742 (1–3), 51–58. doi: 10.1016/j.bbamcr.2004.09.029

Azimi, I., Milevskiy, M. J. G., Kaemmerer, E., Turner, D., Yapa, K. T. D. S., Brown, M. A., et al. (2017). TRPC1 Is a Differential Regulator of Hypoxia-Mediated Events and Akt Signalling in PTEN-Deficient Breast Cancer Cells. *J. Cell Sci.* 130 (14), 2292–2305. doi: 10.1242/jcs.196659

Bader, M. F., Doussau, F., Chasserot-Golaz, S., Vitale, N., and Gasman, S (2004). Coupling Actin and Membrane Dynamics during Calcium-Regulated Exocytosis: A Role for Rho and ARF GTPases. *Biochim. Biophys. Acta - Mol. Cell Res.* 1742 (1–3), 37–49. doi: 10.1016/j.bbamcr.2004.09.028

Baldoli, E., Castiglioni, S., and Maier, J. A.M. (2013). Regulation and Function of TRPM7 in Human Endothelial Cells: TRPM7 as a Potential Novel Regulator of Endothelial Function. *PloS One* 8 (3), e598915. doi: 10.1371/journal.pone.0059891

Bernardini, M., Pla, A. F., Prevarskaya, N., and Gkika, D. (2015). Human Transient Receptor Potential (TRP) Channels Expression Profiling in Carcinogenesis. *Int. J. Dev. Biol.* 59 (7–9), 399–406. doi: 10.1387/ijdb.150232dg

Berridge, M. J., Bootman, M. D., and Roderick, H. L. (2003). CALCIUM SIGNALLING : DYNAMICS, HOMEOSTASIS AND REMODELLING. *Nat. Rev. Mol. Cell Biol.* 4 (7), 517–529. doi: 10.1038/nrm1155

Betson, M., and Braga, V. M.M. (2003). Tumor Progression : Small GTPases and Loss of Cell – Cell Adhesion. *Bioessays* 25 (5), 452–635. doi: 10.1002/bies.10262

Bezzerides, V. J., Ramsey, I. S., Kotecha, S., Greka, A., and Clapham, D. E. (2004). Rapid Vesicular Translocation and Insertion of TRP Channels. *Nat. Cell Biol.* 6 (8), 709–720. doi: 10.1038/ncb1150

Bidaux, G., Roudbaraki, M., Merle, C., Crépin, A., Delcourt, P., Slomianny, C., et al. (2005). Evidence for Specific TRPM8 Expression in Human Prostate Secretory Epithelial Cells: Functional Androgen Receptor Requirement. *Endoc.-Rel. Cancer* 12 (2), 367–382. doi: 10.1677/erc.1.00969

Birukova, A. A., Smurova, K., Birukov, K. G., Kaibuchi, K., Garcia, J. G.N., and Verin, A. D. (2004). Role of Rho GTPases in Thrombin-Induced Lung Vascular Endothelial Cells Barrier Dysfunction. *Microvascular* 67, 64–77. doi: 10.1016/j.mvr.2003.09.007

Boettner, B., and Van Aelst, L. (2009). Control of Cell Adhesion Dynamics by Rap1 Signaling. *Curr. Opin. Cell Biol.* 21 (5), 684–693. doi: 10.1016/j.ceb.2009.06.004

Bomben, V. C., Turner, K. L., Barclay, T. C., and Sontheimer, H. (2011). Transient Receptor Potential Canonical Channels Are Essential for Chemotactic Migration of Human Malignant Gliomas. *J. Cell Physiol.* 226 (7), 1879–1888. doi: 10.1161/CIRCULATIONAHA.110.956839

Bos, J. L. (1989). Ras Oncogenes in Human Cancer: A Review. *Cancer Res.* 49 (17), 4682–4689.

Brossa, A., Buono, L., Fallo, S., Fiorio Pla, A., Munaron, L., and Bussolati, B. (2019). Alternative Strategies to Inhibit Tumor Vascularization. *Int. J. Mol. Sci.* 20 (24), 6180. doi: 10.3390/ijms20246180

Bryan, B. A., and D'Amore, P. A. (2007). What Tangled Webs They Weave: Rho-GTPase Control of Angiogenesis. *Cell. Mol. Life Sci.* 64 (16), 2053–2065. doi: 10.1007/s00018-007-7008-z

Cáceres, M., Ortiz, L., Recabarren, T., Romero, A., Colombo, A., Leiva-Salcedo, E., et al. (2015). TRPM4 Is a Novel Component of the Adhesome Required for Focal Adhesion Disassembly, Migration and Contractility. *PloS One* 10 (6), 1–23. doi: 10.1371/journal.pone.0130540

Cai, N., Lou, L., Al-Saadi, N., Tetteh, S., and Runnels, L. W. (2018). The Kinase Activity of the Channel-Kinase Protein TRPM7 Regulates Stability and Localization of the TRPM7 Channel in Polarized Epithelial Cells. *J. Biol. Chem.* 293 (29), 11491–11504. doi: 10.1074/jbc.RA118.001925

Canales, J., Morales, D., Blanco, C., Rivas, J., Diaz, N., Angelopoulos, I., et al. (2019). A Tr(i)p to Cell Migration: New Roles of Trp Channels in Mechanotransduction and Cancer. *Front. Physiol.* 10:757 (JUN). doi: 10.3389/fphys.2019.00757

Cao, R., Meng, Z., Liu, T. , Wang, G., Qian, G., and Cao, T. (2016). Decreased TRPM7 Inhibits Activities and Induces Apoptosis of Bladder Cancer Cells via ERK1 / 2 Pathway. *Oncotarget* 7 (45), 72941–72960. doi: 10.18632/oncotarget.12146

Cappelli, H. C., Kanugula, A. K., Adapala, R. K., Amin, V., Sharma, P., Midha, P., et al. (2019). Mechanosensitive TRPV4 Channels Stabilize VE-Cadherin

Junctions to Regulate Tumor Vascular Integrity and Metastasis. *Cancer Lett.* 442, 15–20. doi: 10.1016/j.canlet.2018.07.042

Caprodossi, S., Lucciarini, R., Amantini, C., Nabissi, M., Canesin, G., Ballarini, P., et al. (2008). Transient Receptor Potential Vanilloid Type 2 (TRPV2) Expression in Normal Urothelium and in Urothelial Carcinoma of Human Bladder: Correlation with the Pathologic Stage. *Eur. Urol.* 54 (3), 612–620. doi: 10.1016/j.eururo.2007.10.016

Carmeliet, P., and Jain, R. K. (2000). Angiogenesis in Cancer and Other Diseases. *Nature* 407 (6801), 249–257. doi: 10.1038/35025220

Carmeliet, P., and Jain, R. K. (2011). Principles and Mechanisms of Vessel Normalization for Cancer and Other Angiogenic Diseases. *Nat. Rev. Drug Discovery* 10 (6), 417–427. doi: 10.1038/ndr3455

Carmona, G., Go, S., Orlandi, A., Ba, T., Jugold, M., Kiessling, F., et al. (2009). Role of the Small GTPase Rap1 for Integrin Activity Regulation in Endothelial Cells and Angiogenesis. *Blood* 113 (2), 488–497. doi: 10.1182/blood-2008-02-138438

Chen, J. P., Wang, J., Luan, Y., Wang, C. X., Li, W. H., Zhang, J. B., et al. (2015). TRPM7 Promotes the Metastatic Process in Human Nasopharyngeal Carcinoma. *Cancer Lett.* 356 (2), 483–490. doi: 10.1016/j.canlet.2014.09.032

Chen, L., Cao, R., Wang, G., Yuan, L., and Qian, G. (2017). Downregulation of TRPM7 Suppressed Migration and Invasion by Regulating Epithelial – Mesenchymal Transition in Prostate Cancer Cells. *Med. Oncol.* 34 (7), 1–11. doi: 10.1007/s12032-017-0987-1

Chen, Z., Zhu, Y., Dong, Y., Zhang, P., Han, X., Jin, J., et al. (2017). Overexpression of TrpC5 Promotes Tumor Metastasis via the HIF-1α-Twist Signaling Pathway in Colon Cancer. *Clin. Sci.* 131 (19), 2439–2450. doi: 10.1042/CS20171069

Cherfils, J., and Zeghouf, M. (2013). Regulation of Small GTPases by GEFs, GAPs, and GDIs. *Physiol. Rev.* 93 (1), 269–309. doi: 10.1152/physrev.00003.2012

Chigurupati, S., Venkataraman, R., Barrera, D., Naganathan, A., Madan, M., Paul, L., et al. (2010). Receptor Channel TRPC6 Is a Key Mediator of Notch-Driven Glioblastoma Growth and Invasiveness. *Cancer Res.* 6 (11), 418–428. doi: 10.1158/0008-5472.CAN-09-2654

Chrzanowska-wodnicka, M., Kraus, A. E., Gale, D., Ii, G. C.W., and Vansluys, J. (2008). Defective Angiogenesis, Endothelial Migration, Proliferation, and MAPK Signaling in Rap1b-Deficient Mice. *Blood* 111 (5), 2647–2656. doi: 10.1182/blood-2007-08-109710

Chrzanowska-Wodnicka, M. (2010). Regulation of Angiogenesis by a Small GTPase Rap1. *Vasc. Pharmacol.* 53 (1–2), 1–10. doi: 10.1016/j.vph.2010.03.003

Chung, H. K., Rathor, N., Wang, S. R., Wang, J. Y., and Rao, J. N. (2015). RhoA Enhances Store-Operated Ca2+ Entry and Intestinal Epithelial Restitution by Interacting with TRPC1 after Wounding. *Am. J. Physiol. - Gastrointest. Liver Physiol.* 309 (9), G759–G767. doi: 10.1152/ajpgi.00185.2015

Clark, K., Middelbeek, J., Lasonder, E., Dulyaninova, N. G., Morrice, A., Ryazanov, A. G., et al. (2008). TRPM7 Regulates Myosin IIA Filament Stability and Protein Localization by Heavy Chain Phosphorylation. *J. Mol. Biol.* 378 (4), 790–803. doi: 10.1016/j.jmb.2008.02.057

Clayton, N. S., and Ridley, A. J. (2020). Targeting Rho GTPase Signaling Networks in Cancer. *Front. Cell Dev. Biol.* 8:222. doi: 10.3389/fcell.2020.00222

Correll, R. N., Pang, C., Niedowicz, D. M., Finlin, B. S., and Andres, D. A. (2008). The RGK Family of GTP-Binding Proteins: Regulators of Voltage-Dependent Calcium Channels and Cytoskeleton Remodeling. *Cell. Signal.* 20 (2), 292–300. doi: 10.1016/j.cellsig.2007.10.028

Cullen, P. J., and Lockyer, P. J. (2002). Integration of Calcium and Ras Signalling. *Nat. Rev. Mol. Cell Biol.* 3 (5), 339–348. doi: 10.1038/nrm808

Davis, F. M., Azimi, I., Faville, R. A., Peters, A. A., Jalink, K., Putney, J. W. Jr., et al. (2014). Induction of Epithelial – Mesenchymal Transition (EMT) in Breast Cancer Cells Is Calcium Signal Dependent. *Oncogene* 33 (18), 2307–2316. doi: 10.1038/onc.2013.187

Desai, B. N., Krapivinsky, G., Navarro, B., Krapivinsky, L., Carter, B. C., Febvay, S., et al. (2012). Cleavage of TRPM7 Releases the Kinase Domain from the Ion Channel and Regulates Its Participation in Fas-Induced Apoptosis. *Dev. Cell* 22 (6), 1149–1162. doi: 10.1016/j.devcel.2012.04.006

Dhennin-duthille, I., Gautier, M., Faouzi, M., Brevet, M., Vaudry, D., Ahidouch, A., et al. (2011). Cellular Physiology and Biochemistr y Biochemistry High Expression of Transient Receptor Potential Channels in Human Breast Cancer Epithelial Cells and Tissues: Correlation with Pathological Parameters. *Cell Physiol. Biochem.* 28, 813–822.

Dong, H., Shim, K. N., Li, J. M. J., Estrema, C., Ornelas, T. A., Nguyen, F., et al. (2010). Molecular Mechanisms Underlying Ca2+-Mediated Motility of Human Pancreatic Duct Cells. *Am. J. Physiol. - Cell Physiol.* 299 (6), 1493–1503. doi: 10.1152/ajpcell.00242.2010

Elbaz, M., Ahirwar, D., Xiaoli, Z., Zhou, X., Lustberg, M., Nasser, M. W., et al. (2018). TRPV2 Is a Novel Biomarker and Therapeutic Target in Triple Negative Breast Cancer. *Oncotarget* 9 (71), 33459–33470. doi: 10.18632/oncotarget.9663

Fabian, A., Fortmann, T., Dieterich, P., Riethmüller, C., Schön, P., Mally, S., et al. (2008). TRPC1 Channels Regulate Directionality of Migrating Cells. *Pflugers Archiv. Eur. J. Physiol.* 457 (2), 475–484. doi: 10.1007/s00424-008-0515-4

Faouzi, M., Kilch, T., Horgen, F. D., Fleig, A., and Penner, R. (2017). The TRPM7 Channel Kinase Regulates Store-Operated Calcium Entry. *J. Physiol.* 595 (10), 3165–3180. doi: 10.1113/JP274006

Fels, B., Bulk, E., Pethő, Z., and Schwab, A. (2018). The Role of TRP Channels in the Metastatic Cascade. *Pharmaceuticals* 11 (2), 48. doi: 10.3390/ph11020048

Fiorio Pla, A. F., and Gkika, D. (2013). Emerging Role of TRP Channels in Cell Migration : From Tumor Vascularization to Metastasis. *Front. Physiol.* 4, 1–12. doi: 10.3389/fphys.2013.00311

Fiorio Pla, A., Ong, H. L., Cheng, K. T., Brossa, A., Bussolati, B., Lockwich, T., et al. (2012). TRPV4 Mediates Tumor-Derived Endothelial Cell Migration via Arachidonic Acid-Activated Actin Remodeling. *Oncogene* 31 (2), 200–212. doi: 10.1016/j.physbeh.2017.03.040

Fuessel, S., Sickert, D., Meye, A., Klenk, U., Schmidt, U., Schmitz, M., et al. (2003). Multiple Tumor Marker Analyses (PSA, HK2, PSCA, Trp-P8) in Primary Prostate Cancers Using Quantitative RT-PCR. *Int. J. Oncol.* 23 (1), 221–228. doi: 10.3892/ijo.23.1.221

Gao, G., Wang, W., Tadagavadi, R. K., Briley, N. E., Love, M.II, Miller, B. A., et al. (2014). TRPM2 Mediates Ischemic Kidney Injury and Oxidant Stress through RAC1. *J. Clin. Invest.* 124 (11), 4989–5001. doi: 10.1172/JCI76042.us

Genova, T., Grolez, G. P., Camillo, C., Bernardini, M., Bokhobza, A., Richard, E., et al. (2017). TRPM8 Inhibits Endothelial Cell Migration via a Nonchannel Function by Trapping the Small GTPase Rap1. *J. Cell Biol.* 216 (7), 2107–2130. doi: 10.1083/jcb.201506024

Ghosh, K., Thodeti, C. K., Dudley, A. C., Mammoto, A., Klagsbrun, M., and Ingber, D. E. (2008). Tumor-Derived Endothelial Cells Exhibit Aberrant Rho-Mediated Mechanosensing and Abnormal Angiogenesis in Vitro. *Proc. Natl. Acad. Sci. U. S. A.* 105 (32), 11305–11310. doi: 10.1073/pnas.0800835105

Gkika, D., and Prevarskaya, N. (2011). TRP Channels in Prostate Cancer: The Good, the Bad and the Ugly? *Asian J. Androl.* 13 (5), 673–676. doi: 10.1038/aja.2011.18

Greka, A., Navarro, B., Oancea, E., Duggan, A., and Clapham, D. E. (2003). TRPC5 Is a Regulator of Hippocampal Neurite Length and Growth Cone Morphology. *Nat. Neurosci.* 6 (8), 837–845. doi: 10.1038/nn1092

Guéguinou, M., Harnois, T., Crottes, D., Uguen, A., Deliot, N., Gambade, A., et al. (2016). SK3/TRPC1/Orai1 Complex Regulates SOCE-Dependent Colon Cancer Cell Migration: A Novel Opportunity to Modulate Anti- EGFR MAb Action by the Alkyl-Lipid Ohmline. *Oncotarget* 7 (24), 36168–36184. doi: 10.18632/oncotarget.8786

Guilbert, A., Dhennin-Duthille, I., Hiani, Y. E. L., Haren, N., Khorsi, H., Sevestre, H., et al. (2008). Expression of TRPC6 Channels in Human Epithelial Breast Cancer Cells. *BMC Cancer* 8, 1–11. doi: 10.1186/1471-2407-8-125

Guilbert, A., Gautier, M., Dhennin-Duthille, I., Rybarczyk, P., Sahni, J., Sevestre, H., et al. (2013). Transient Receptor Potential Melastatin 7 Is Involved in Oestrogen Receptor-Negative Metastatic Breast Cancer Cells Migration through Its Kinase Domain. *Eur. J. Cancer* 49 (17), 3694–3707. doi: 10.1016/j.ejca.2013.07.008

Haws, H. J., Mcneil, M. A., and Hansen, M. D.H. (2016). Control of Cell Mechanics by RhoA and Calcium Fl Uxes during Epithelial Scattering. *Tissue Barriers* 4 (3), 1–155. doi: 10.1080/21688370.2016.1187326

Hicks, K., Neil, R. G.O., Dubinsky, W. S., Brown, R. C., Hicks, K., O Neil, R. G., et al. (2010). TRPC-Mediated Actin-Myosin Contraction Is Critical for BBB Disruption Following Hypoxic Stress. *Am. J. Physiol. Cell Physiol.* 298, 1583–1593. doi: 10.1152/ajpcell.00458.2009

Ho, H.-h., Chang, C.-s., Ho, W.-c., Liao, S.-y., and Wu, C.-h. (2010). Anti-Metastasis Effects of Gallic Acid on Gastric Cancer Cells Involves Inhibition of NF- j B Activity and Downregulation of PI3K / AKT / Small GTPase Signals. *Food Chem. Toxicol.* 48 (8–9), 2508–2516. doi: 10.1016/j.fct.2010.06.024

Hobbs, G. A., Der, C. J., and Rossman, K. L. (2016). RAS Isoforms and Mutations in Cancer at a Glance. *J. Cell Sci.* 129 (7), 1287–1292. doi: 10.1242/jcs.182873

Holzmann, C., Kappel, S., Kilch, T., Jochum, M. M., Urban, S. K., Jung, V., et al. (2015). Transient Receptor Potential Melastatin 4 Channel Contributes to Migration of Androgen-Insensitive Prostate Cancer Cells. *Oncotarget* 6 (39), 41783–41793. doi: 10.18632/oncotarget.6157

Huang, Y. W., Chang, S. J., Harn, H.II, Huang, H. T., Lin, H. H., Shen, M. R., et al. (2015). Mechanosensitive Store-Operated Calcium Entry Regulates the Formation of Cell Polarity. *J. Cell. Physiol.* 230 (9), 2086–2097. doi: 10.1002/jcp.24936

Iamshanova, O., Pla, A. F., and Prevarskaya, N. (2017). Molecular Mechanisms of Tumour Invasion: Regulation by Calcium Signals. *J. Physiol.* 595 (10), 3063–3075. doi: 10.1113/JP272844

Iftinca, M., Hamid, J., Chen, L., Varela, D., Tadayonnejad, R., Altier, C., et al. (2007). Regulation of T-Type Calcium Channels by Rho-Associated Kinase. *Nat. Neurosci.* 10 (7), 854–860. doi: 10.1038/nn1921

Jabłońska-trypuć, A., Matejczyk, M., and Rosochacki, S. (2016). Matrix Metalloproteinases (MMPs), the Main Extracellular Matrix (ECM) Enzymes in Collagen Degradation, as a Target for Anticancer Drugs Enzymes in Collagen Degradation, as a Target for Anticancer Drugs. *J. Enzyme Inhib. Med. Chem.* 31 (sup1), 177–183. doi: 10.3109/14756366.2016.1161620

Jacob, A., Jing, J., Lee, J., Schedin, P., Gilbert, S. M., Peden, A. A., et al. (2013). Rab40b Regulates Trafficking of MMP2 and MMP9 during Invadopodia Formation and Invasion of Breast Cancer Cells. *J. Cell Sci.* 126 (Pt20), 4647–4658. doi: 10.1242/jcs.126573

Jiang, L. H., Gamper, N., and Beech, D. J. (2011). Properties and Therapeutic Potential of Transient Receptor Potential Channels with Putative Roles in Adversity: Focus on TRPC5, TRPM2 and TRPA1. *Curr. Drug Targets* 12 (5), 724–736. doi: 10.2174/138945011795378568

Kim, J. G., Islam, R., Cho, J. Y., Jeong, H., Cap, K. C., Park, Y., et al. (2018). Regulation of RhoA GTPase and Various Transcription Factors in the RhoA Pathway. *J. Cell. Physiol.* 233 (9), 6381–6392. doi: 10.1002/jcp.26487

Koike, T., Kuzuya, M., Asai, T., Kanda, S., and Cheng, X. W. (2000). Activation of MMP-2 by Clostridium Difficile Toxin B in Bovine Smooth Muscle Cells. *Biochem. Biophys. Res. Commun.* 46, 43–46. doi: 10.1006/bbrc.2000.3630

Koopman, W. J. H., Bosch, R. R., Van Emst-De Vries, S. E., Spaargaren, M., De Pont Jan Joep, H. H. M., and Willems, P. H. G. M. (2003). R-Ras Alters Ca2+ Homeostasis by Increasing the Ca2+ Leak across the Endoplasmic Reticular Membrane. *J. Biol. Chem.* 278 (16), 13672–13679. doi: 10.1074/jbc.M211256200

Laragione, T., Cheng, K. F., Tanner, M. R., He, M., Beeton, C., Al-Abed, Y., et al. (2015). THE CATION CHANNEL TRPV2 IS A NEW SUPPRESSOR OF ARTHRITIS SEVERITY, JOINT DAMAGE AND SYNOVIAL FIBROBLAST INVASION. *Clin. Immunol.* 158 (2), 183–192. doi: 10.1016/j.clim.2015.04.001

Laragione, T., Harris, C., and Gulko, P. S. (2019). TRPV2 Suppresses Rac1 and RhoA Activation and Invasion in Rheumatoid Arthritis Fibroblast-like Synoviocytes. *Int. Immunopharmacol.* 70, 268–273. doi: 10.1016/j.intimp.2019.02.051

Lastraioli, E., Iorio, J., and Arcangeli, A. (2015). Ion Channel Expression as Promising Cancer Biomarker. *Biochim. Biophys. Acta - Biomembr.* 1848 (10), 2685–2702. doi: 10.1016/j.bbamem.2014.12.016

Lee, W. H., Choong, L. Y., Mon, N. N., Lu, S., Lin, Q., Pang, B., et al. (2016). "TRPV4 Regulates Breast Cancer Cell Extravasation, Stiffness and Actin Cortex. *Sci. Rep.* 6 (May), 1–16. doi: 10.1038/srep27903

Lee, W. H., Choong, L. Y., Jin, T. H., Mon, N. N., Chong, S., Liew, C. S., et al. (2017). TRPV4 Plays a Role in Breast Cancer Cell Migration via Ca2+-Dependent Activation of AKT and Downregulation of E-Cadherin Cell Cortex Protein. *Oncogenesis* 6 (5), e338. doi: 10.1038/oncsis.2017.39

Lehen'kyi, V., and Prevarskaya, N. (2011). "Oncogenic TRP Channels." in *Transient Receptor Potential Channels. Advances in Experimental Medicine and Biology*. Ed. M. Islam (United States: Springer), 929–945.

Lepannetier, S., Zanou, N, Yerna, X., Emeriau, N., Dufour, I., Masquelier, J., et al. (2016). Sphingosine-1-Phosphate-Activated TRPC1 Channel Controls Chemotaxis of Glioblastoma Cells. *Cell Calcium* 60 (6), 373–383. doi: 10.1016/j.ceca.2016.09.002

Li, S., Balmain, A., and Counter, C. M. (2018). A Model for RAS Mutation Patterns in Cancers: Finding the Sweet Spot. *Nat. Rev. Cancer* 18 (12), 767–777. doi: 10.1038/s41568-018-0076-6

Litan, A., and Langhans, S. A. (2015). Cancer as a Channelopathy: Ion Channels and Pumps in Tumor Development and Progression. *Front. Cell. Neurosci.* 9:86 (March). doi: 10.3389/fncel.2015.00086

Liu, J., Chen, Y., Shuai, S., and Ding, D. (2014). TRPM8 Promotes Aggressiveness of Breast Cancer Cells by Regulating EMT via Activating AKT / GSK-3 β Pathway. *Tumour Biol.* 35 (9), 8969–8977. doi: 10.1007/s13277-014-2077-8

Liu, K., Xu, S.-h., Chen, Z., Zeng, Q.-x., Li, Z.-j., and Chen, Z.-m. (2018). TRPM7 Overexpression Enhances the Cancer Stem Cell-like and Metastatic Phenotypes of Lung Cancer through Modulation of the Hsp90 α / UPA / MMP2 Signaling Pathway. *BMC Cancer* 18 (1), 1167. doi: 10.1186/s12885-018-5050-x

Liu, L., Wu, N., Wang, Y., Zhang, X., Xia, B., Tang, J., et al. (2019). TRPM7 Promotes the Epithelial – Mesenchymal Transition in Ovarian Cancer through the Calcium-Related PI3K / AKT Oncogenic Signaling. *J. Exp. Clin. Cancer Res.* 38 (1), 106–121. doi: 10.1186/s13046-019-1061-y

Llense, F., and Etienne-Manneville, S. (2015). "Front-to-Rear Polarity in Migrating Cells." in *Cell Polarity 1*. Ed. E. Klaus (United States: Springer), 115–146. doi: 10.1007/978-3-319-14463-4_5

Luo, Y., Wu, J.-y., Lu, M.-h., Shi, Z., Na, N., and Di, J.-m. (2016). Carvacrol Alleviates Prostate Cancer Cell Proliferation, Migration, and Invasion through Regulation of PI3K / Akt and MAPK Signaling Pathways. *Oxid. Med. Cell Longev.* 2016, 1469693. doi: 10.1155/2016/1469693

Mehta, D., Ahmmed, G. U., Paria, B. C., Holinstat, M., Voyno-Yasenetskaya, T., Tiruppathi, C., et al. (2003). RhoA Interaction with Inositol 1,4,5-Trisphosphate Receptor and Transient Receptor Potential Channel-1 Regulates Ca2+ Entry: Role in Signaling Increased Endothelial Permeability. *J. Biol. Chem.* 278 (35), 33492–33500. doi: 10.1074/jbc.M302401200

Meng, X., Cai, C., Wu, J., Cai, S., Ye, C., Chen, H., et al. (2013). TRPM7 Mediates Breast Cancer Cell Migration and Invasion through the MAPK Pathway. *Cancer Lett.* 333 (1), 96–102. doi: 10.1016/j.canlet.2013.01.031

Middelbeek, J., Kuipers, A. J., Henneman, L., Visser, D., Eidhof, I., Van Horssen, R., et al. (2012). TRPM7 Is Required for Breast Tumor Cell Metastasis. *Cancer Res.* 72 (16), 4250–4261. doi: 10.1158/0008-5472.CAN-11-3863

Mishra, A. K., and Lambright, D. G. (2016). Small GTPases and Their GAPs. *Biopolymers* 105 (8), 431–448. doi: 10.1002/bip.22833

Moissoglu, K., and Schwartz, M. A. (2014). Spatial and Temporal Control of Rho GTPase Functions. *Cell. Logistics* 4 (2), e943618. doi: 10.4161/21592780.2014.943618

Monet, M., Lehen, V., Gackiere, F., Firlej, V., Vandenberghe, M., Roudbaraki, M., et al. (2010). Role of Cationic Channel TRPV2 in Promoting Prostate Cancer Migration and Progression to Androgen Resistance. *Cancer Res.* 70 (3), 1225–1236. doi: 10.1158/0008-5472.CAN-09-2205

Monteith, G. R., Prevarskaya, N., and Roberts-Thomson, S. J. (2017). The Calcium–cancer Signalling Nexus. *Nat. Rev. Cancer* 17 (6), 367–380. doi: 10.1038/nrc.2017.18

Mrkonjić, S., Garcia-Elias, A., Pardo-Pastor, C., Bazellières, E., Trepat, X., Vriens, J., et al. (2015). TRPV4 Participates in the Establishment of Trailing Adhesions and Directional Persistence of Migrating Cells. *Pflugers Archiv. Eur. J. Physiol.* 467 (10), 2107–2119. doi: 10.1007/s00424-014-1679-8

Nagasawa, M., and Kojima, I. (2015). Translocation of TRPV2 Channel Induced by Focal Administration of Mechanical Stress. *Physiol. Rep.* 3, 1–12. doi: 10.14814/phy2.12296

Narayan, G., Bourdon, V., Chaganti, S., Arias-Pulido, H., Nandula, S. V., Rao, P. H., et al. (2007). Gene Dosage Alterations Revealed by CDNA Microarray Analysis in Cervical Cancer: Identification of Candidate Amplified and Overexpressed Genes. *Genes Chromosomes Cancer* 46 (4), 373–384. doi: 10.1002/gcc.20418

Negri, S., Faris, P., Berra-Romani, R., Guerra, G., and Moccia, F. (2020). Endothelial Transient Receptor Potential Channels and Vascular Remodeling: Extracellular Ca2 + Entry for Angiogenesis, Arteriogenesis and Vasculogenesis. *Front. Physiol.* 10, 1618 (January). doi: 10.3389/fphys.2019.01618

Nilius, B., and Owsianik, G. (2011). The Transient Receptor Potential Family of Ion Channels. *Genome Biol.* 12 (3), 218. doi: 10.1186/gb-2011-12-3-218

Okamoto, Y., Ohkubo, T., Ikebe, T., and Yamazaki, J. U. N. (2012). Blockade of TRPM8 Activity Reduces the Invasion Potential of Oral Squamous Carcinoma Cell Lines. *Int. J. Oncol.* 40 (5), 1431–1440. doi: 10.3892/ijo.2012.1340

Oulidi, A., Bokhobza, A., Gkika, D., Vanden Abeele, F., Lehen, V., Houcine Ouafik, L., et al. (2013). TRPV2 Mediates Adrenomedullin Stimulation of Prostate and Urothelial Cancer Cell Adhesion, Migration and Invasion. *PLoS One* 8 (5), 1–7. doi: 10.1371/journal.pone.0064885

Ou-yang, Q., Li, B., Xu, M., and Liang, H. (2018). TRPV4 Promotes the Migration and Invasion of Glioma Cells via AKT/Rac1 Signaling. *Biochem. Biophys. Res. Commun.* 503 (2), 876–881. doi: 10.1016/j.bbrc.2018.06.090

Prior, I. A., Lewis, P. D., and Mattos, C. (2012). A Comprehensive Survey of Ras Mutations in Cancer. *Cancer Res.* 72 (10), 2457–2467. doi: 10.1158/0008-5472.CAN-11-2612.A

Rampino, T., Gregorini, M., Guidetti, C., Broggini, M., Marchini, S., Bonomi, R., et al. (2007). KCNA1 and TRPC6 Ion Channels and NHE1 Exchanger Operate the Biological Outcome of HGF / Scatter Factor in Renal Tubular Cells. *Growth Factors* 25, 382–391. doi: 10.1080/08977190801892184

Ridley, A. J. (2001). Rho Family Proteins : Coordinating Cell Responses. *Growth Factors* 11 (12), 471–477. doi: 10.1016/s0962-8924(01)02153-5

Roche, J. (2018). The Epithelial-to-Mesenchymal Transition in Cancer. *Cancers* 10 (3), 10–13. doi: 10.3390/cancers10020052

Rybarczyk, P., Gautier, M., Hague, F., Dhennin-Duthille, I., Chatelain, D., Kerr-Conte, J., et al. (2012). Transient Receptor Potential Melastatin-Related 7 Channel Is Overexpressed in Human Pancreatic Ductal Adenocarcinomas and Regulates Human Pancreatic Cancer Cell Migration. *Int. J. Cancer* 131 (6), 851–861. doi: 10.1002/ijc.27487

Rybarczyk, P., Vanlaeys, A., Brassart, B., Dhennin-duthille, I., Chatelain, D., Sevestre, H., et al. (2017). The Transient Receptor Potential Melastatin 7 Channel Regulates Pancreatic Cancer Cell Invasion through the Hsp90 α / UPA / MMP2. *Neoplasia* 19 (4), 288–300. doi: 10.1016/j.neo.2017.01.004

Sahai, E., and Marshall, C. J. (2002). RHO - GTPases and Cancer. *Nat. Rev. Cancer* 2 (2), 133–142. doi: 10.1038/nrc725

Santibáñez, J. F., Kocic, J., Fabra, A., Cano, A., and Quintanilla, M. (2010). Rac1 Modulates TGF- b 1-Mediated Epithelial Cell Plasticity and MMP9 Production in Transformed Keratinocytes. *FEBS Lett.* 584 (11), 2305–2310. doi: 10.1016/j.febslet.2010.03.042

Scarpellino, S., Munaron, L., Cantelmo, A. R., and Fiorio Pla, A. (2020). Calcium-Permeable Channels in Tumor Vascularization: Peculiar Sensors of Microenvironmental Chemical and Physical Cues. *Rev. Physiol. Biochem. Pharmacol.* doi: 10.1007/112_2020_32

Seiz, J. R., Klinke, J., Scharlibbe, L., Lohfink, D., and Heipel, M. (2020). Different Signaling and Functionality of Rac1 and Rac1b in the Progression of Lung Adenocarcinoma. *Biol. Chem.* 401 (4), 517–531. doi: 10.1515/hsz-2019-0329

Shapovalov, G., Ritaine, A., Skryma, R., and Prevarskaya, N. (2016). Role of TRP Ion Channels in Cancer and Tumorigenesis. *Semin. Immunopathol.* 38 (3), 357–369. doi: 10.1007/s00281-015-0525-1

Shields, J. M., Pruitt, K., Shaub, A., and Der, C. J. (2000). Understanding Ras : ' It Ain ' t over ' Til It ' s over '. *Trends Cell Biol.* 10 (4), 147–154. doi: 10.1016/s0962-8924(00)01740-2

Simon, A. R., Vikis, H. G., Steward, S., Fanburg, B. L., Cochran, B. H., and Guan, K.-L. (2000). Regulation of STAT3 by Direct Binding to the Racl GTPase. *Science* 290 (5489), 144–147. doi: 10.1126/science.290.5489.144

Singh, J., Manickam, P., Shmoish, M., Natik, S., Denyer, G., Handelsman, D., et al. (2006). Annotation of Androgen Dependence to Human Prostate Cancer-Associated Genes by Microarray Analysis of Mouse Prostate. *Cancer Lett.* 237 (2), 298–304. doi: 10.1016/j.canlet.2005.06.008

Singh, I., Knezevic, N., Ahmmed, G. U., Kini, V., Malik, A. B., and Mehta, D. (2007). Gαq -TRPC6-Mediated Ca 2+ Entry Induces RhoA Activation and Resultant Endothelial Cell Shape Change in Response to Thrombin. *J. Biol. Chem.* 282 (11), 7833–7843. doi: 10.1074/jbc.M608288200

Sokabe, T., and Tominaga, M. (2010). The TRPV4 Cation Channel. *Communicative Integr. Biol.* 3 (6), 619–621. doi: 10.4161/cib.3.6.13461

Stenmark, H. (2009). Rab GTPases as Coordinators of Vesicle Traffic. *Nat. Rev. Mol. Cell Biol.* 10 (8), 513–525. doi: 10.1038/nrm2728

Su, L.-t., Liu, W., Chen, H.-c., An, O. G. A.-p., Habas, R., Runnels, L. W., et al. (2011). TRPM7 Regulates Polarized Cell Movements. *Biochem. J.* 434 (3), 513–521. doi: 10.1042/BJ20101678

Sumoza-Toledo, A., Espinoza-Gabriel, M.II, and Montiel-Condado, D. (2016). Evaluación Del Canal TRPM2 Como Biomarcador En Cáncer de Mama Mediante El Análisis de Bases de Datos Públicos. *Boletin Medico Del Hosp. Infantil Mexico* 73 (6), 397–404. doi: 10.1016/j.bmhimx.2016.10.001

Sun, J., Yang, T., Wang, P., Ma, S., Zhu, Z., Pu, Y., et al. (2014). Activation of Cold-Sensing Transient Receptor Potential Melastatin Subtype 8 Antagonizes Vasoconstriction and Hypertension through Attenuating RhoA/Rho Kinase Pathway. *Hypertension* 63 (6), 1354–1363. doi: 10.1161/HYPERTENSIONAHA.113.02573

Svensmark, J. H., and Brakebusch, C. (2019). Rho GTPases in Cancer: Friend or Foe? *Oncogene* 38 (50), 7447–7456. doi: 10.1038/s41388-019-0963-7

Thodeti, C. K., Matthews, B., Ravi, A., Mammoto, A., Ghosh, K., Bracha, A. L., et al. (2009). TRPV4 Channels Mediate Cyclic Strain-Induced Endothelial Cell Reorientation through Integrin-to-Integrin Signaling. *Circ. Res.* 104 (9), 1123–1130. doi: 10.1161/CIRCRESAHA.108.192930

Thodeti, C. K., Paruchuri, S., and Meszaros, J. G. (2013). A TRP to Cardiac Fibroblast Differentiation. *Channels* 7 (3), 211–214. doi: 10.4161/chan.24328

Thoppil, R. J., Cappelli, H. C., Adapala, R. K., Kanugula, A. K., Paruchuri, S., and Thodeti, C. K. (2016). TRPV4 Channels Regulate Tumor Angiogenesis via Modulation of Rho/Rho Kinase Pathway. *Oncotarget* 7 (18), 25849–25861. doi: 10.18632/oncotarget.8405

Tian, D., Jacobo, S. M.P., Billing, D., Rozkalne, A., Gage, S. D., Anagnostou, T., et al. (2010). Antagonistic Regulation Od Actin Dynamics and Cell Motility by TRPC5 and TRPC6 Channels. *Sci. Signal.* 3 (145), 1–25. doi: 10.1126/scisignal.2001200.Antagonistic

Tomasek, J. J., Vaughan, M. B., Kropp, B. P., Gabbiani, G., Martin, M. D., and Hinz, B. (2006). Contraction of Myofibroblasts in Granulation Tissue Is Dependent on Rho / Rho Kinase / Myosin Light Chain Phosphatase Activity. *Wound Repair Regen.* 14 (3), 313–320. doi: 10.1111/j.1743-6109.2006.00126.x

Tsavaler, L., Shapero, M. H., Morkowski, S., and Laus, R. (2001). Trp-P8, a Novel Prostate-Specific Gene, Is up-Regulated in Prostate Cancer and Other Malignancies and Shares High Homology with Transient Receptor Potential Calcium Channel Proteins. *Cancer Res.* 61 (9), 3760–3769.

Ungefroren, H., Witte, D., and Lehnert, H. (2018). The Role of Small GTPases of the Rho/Rac Family in TGF-β-Induced EMT and Cell Motility in Cancer. *Dev. Dynamics* 247 (3), 451–461. doi: 10.1002/dvdy.24505

Vennekens, R., and Nilius, B. (2007). "Insights into TRPM4 Function, Regulation and Physiological Role." in *Transient Receptor Potential (TRP) Channels. Handbook of Experimental Pharmacology*. Eds. V. Flockerzi and B. Nilius (United States: Springer), 269–285.

Vetter, I. R., and Wittinghofer, A. (2001). The Guanine Nucleotide-Binding Switch in Three Dimensions. *Science* 294 (5545), 1299–1304. doi: 10.1126/science.1062023

Vrenken, K. S., Jalink, K., van Leeuwen, F. N., and Middelbeek, J. (2015). Beyond Ion-Conduction: Channel-Dependent and -Independent Roles of TRP Channels during Development and Tissue Homeostasis. *Biochim. Biophys. Acta - Mol. Cell Res.* 1863 (6), 1436–1446. doi: 10.1016/j.bbamcr.2015.11.008

Vriens, J., Janssens, A., Prenen, J., Nilius, B., and Wondergem, R. (2004). TRPV Channels and Modulation by Hepatocyte Growth Factor / Scatter Factor in Human Hepatoblastoma (HepG2) Cells. *Cell Calcium* 36 (1), 19–28. doi: 10.1016/j.ceca.2003.11.006

Wang, Y., He, H., Srivastava, N., Vikarunnessa, S., Chen, Y. B., Jiang, J., et al. (2012). Plexins Are GTPase-Activating Proteins for Rap and Are Activated by Induced Dimerization. *Sci. Signaling* 5 (207), 1–25. doi: 10.1126/scisignal.2002636

Wei, C., Wang, X., Chen, M., Ouyang, K., Song, L.-S., and Cheng, H. (2009). Calcium Flickers Steer Cell Migration. *Nature* 457 (7231), 901–905. doi: 10.1038/nature07577.Calcium

Xiao, N., Jiang, L. M., Ge, B., Zhang, T. Y., Zhao, X. K., and Zhou, X. (2014). Over-Expression of TRPM8 Is Associated with Poor Prognosis in Urothelial Carcinoma of Bladder. *Tumor Biol.* 35 (11), 11499–11504. doi: 10.1007/s13277-014-2480-1

Xie, R., Xu, J., Xiao, Y., Wu, J., Wan, H., Tang, B., et al. (2017). Calcium Promotes Human Gastric Cancer via a Novel Coupling of Calcium-Sensing Receptor and TRPV4 Channel. *Cancer Res.* 77 (23), 6499–6512. doi: 10.1158/0008-5472.CAN-17-0360

Xu, S.-z., Muraki, K., Zeng, F., Li, J., Sukumar, P., Dedman, A. M., et al. (2006). A Sphingosine-1 – Phosphate – Activated Calcium Channel Controlling Vascular Smooth Muscle Cell Motility. *Cell. Pharmacol.* 98 (11), 1381–1389. doi: 10.1161/01.RES.0000225284.36490.a2.A

Yang, D., and Kim, J. (2020). Emerging Role of Transient Receptor Potential (TRP) Channels in Cancer Progression. *BMB Rep.* 53 (3), 125–132. doi: 10.5483/BMBRep.2020.53.3.016

Yang, J., Liu, W., Lu, X., Fu, Y., Li, L., and Luo, Y. (2015). High Expression of Small GTPase Rab3D Promotes Cancer Progression and Metastasis. *Oncotarget* 6 (13), 11125–11138. doi: 10.18632/oncotarget.3575

Yarwood, S., Cullen, P. J., and Kupzig, S. (2006). The GAP1 Family of GTPase-Activating Proteins : Spatial and Temporal Regulators of Small

GTPase Signalling. *Biochem. Soc. Trans.* 34 (5), 846–850. doi: 10.1042/BST0340846

Yee, N. S., Kazi, A. A., Li, Q., Yang, Z., Berg, A., and Yee, R. K. (2015). Aberrant Over-Expression of TRPM7 Ion Channels in Pancreatic Cancer: Required for Cancer Cell Invasion and Implicated in Tumor Growth and Metastasis. *Biol. Open* 4 (4), 507–514. doi: 10.1242/bio.20137088

Yue, D., Wang, Y., Xiao, J. Y., Wang, P., and Ren, C. S. (2009). Expression of TRPC6 in Benign and Malignant Human Prostate Tissues. *Asian J. Androl.* 11 (5), 541–547. doi: 10.1038/aja.2009.53

Zahra, F. T., Sajib, Md S., Ichiyama, Y., Akwii, R. G., Tullar, P. E., Cobos, C., et al. (2019). Endothelial RhoA GTPase Is Essential for in Vitro Endothelial Functions but Dispensable for Physiological in Vivo Angiogenesis. *Sci. Rep.* 9 (1), 1–15. doi: 10.1038/s41598-019-48053-z

Zeng, Z., Leng, T., Feng, X., Sun, H., Inoue, K., Zhu, L., et al. (2015). Silencing TRPM7 in Mouse Cortical Astrocytes Impairs Cell Proliferation and Migration via ERK and JNK Signaling Pathways. *PloS One* 10 (3), 1–19. doi: 10.1371/journal.pone.0119912

Zhang, B., Zhang, Y., Wang, Z. X., and Zheng, Y. (2000). The Role of Mg2+ Cofactor in the Guanine Nucleotide Exchange and GTP Hydrolysis Reactions of Rho Family GTP-Binding Proteins. *J. Biol. Chem.* 275 (33), 25299–25307. doi: 10.1074/jbc.M001027200

Zhang, S. S., Wen, J., Yang, F., Cai, X. L., Yang, H., Luo, K. J., et al. (2013). High Expression of Transient Potential Receptor C6 Correlated with Poor Prognosis in Patients with Esophageal Squamous Cell Carcinoma. *Med. Oncol.* 30 (3), 607. doi: 10.1007/s12032-013-0607-7

Zhao, W., and Xu, H. (2016). High Expression of TRPM8 Predicts Poor Prognosis in Patients with Osteosarcoma. *Oncol. Lett.* 12 (2), 1373–1379. doi: 10.3892/ol.2016.4764

Zhou, K., Zhang, S. S., Yan, Y., and Zhao, S. (2014). Overexpression of Transient Receptor Potential Vanilloid 2 Is Associated with Poor Prognosis in Patients with Esophageal Squamous Cell Carcinoma. *Med. Oncol.* 31 (7), 17. doi: 10.1007/s12032-014-0017-5

Zhuge, Y., and Xu, J. (2001). Rac1 Mediates Type I Collagen-Dependent MMP-2 Activation. *J. Biol. Chem.* 276 (19), 16248–16256. doi: 10.10974/jbc.M010 90200

Zoppoli, P., Calice, G., Laurino, S., Ruggieri, V., La Rocca, F., La Torre, G., et al. (2019). TRPV2 Calcium Channel Gene Expression and Outcomes in Gastric Cancer Patients: A Clinically Relevant Association. *J. Clin. Med.* 8 (5):662. doi: 10.3390/jcm8050662

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 Chinigò, Fiorio Pla and Gkika. 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 Pharmacology

REVIEW
published: 29 September 2020
doi: 10.3389/fphar.2020.525020

Interplay Between Ion Channels and the Wnt/β-Catenin Signaling Pathway in Cancers

Raphael Rapetti-Mauss, Camille Berenguier, Benoit Allegrini and Olivier Soriani*

Inserm, CNRS, iBV, Université Côte d'Azur, Nice, France

Increasing evidence point out the important roles of ion channels in the physiopathology of cancers, so that these proteins are now considered as potential new therapeutic targets and biomarkers in this disease. Indeed, ion channels have been largely described to participate in many hallmarks of cancers such as migration, invasion, proliferation, angiogenesis, and resistance to apoptosis. At the molecular level, the development of cancers is characterised by alterations in transduction pathways that control cell behaviors. However, the interactions between ion channels and cancer-related signaling pathways are poorly understood so far. Nevertheless, a limited number of reports have recently addressed this important issue, especially regarding the interaction between ion channels and one of the main driving forces for cancer development: the Wnt/β-catenin signaling pathway. In this review, we propose to explore and discuss the current knowledge regarding the interplay between ion channels and the Wnt/β-catenin signaling pathway in cancers.

Keywords: signaling pathways, drug targets, KCNQ1 channel, P2X7, cystic fibrosis transmembrane conductance regulator, hERG, transient receptor protein channels, ClC-2 channel

OPEN ACCESS

Edited by:
Sébastien Roger, Université de Tours, France

Reviewed by:
Irene Frischauf, Johannes Kepler University of Linz, Austria
Heike Wulff, University of California, Davis, United States

****Correspondence:***
Raphael Rapetti-Mauss
Raphael.RAPETTI-MAUSS@univ-cotedazur.fr

Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *07 January 2020*
Accepted: *31 August 2020*
Published: *29 September 2020*

Citation:
Rapetti-Mauss R, Berenguier C, Allegrini B and Soriani O (2020) Interplay Between Ion Channels and the Wnt/β-Catenin Signaling Pathway in Cancers. Front. Pharmacol. 11:525020. doi: 10.3389/fphar.2020.525020

INTRODUCTION

Despite decades of intensive research on its physiopathology, cancer remains a leading cause of death worldwide according to the World Health Organisation. Traditional cancer treatments including radiation therapy, chemotherapy, and surgery have substantially improved patients' conditions. However, several challenges in curing cancer remain in front of us. Side effects generated by cancer treatments significantly impact patients quality of life; high patients intrinsic heterogeneity revealed treatments resistance, and finally the efficiency of treatment used for some types of cancer remains very low such as for pancreatic cancer. These circumstances highlight the urgent need to identify new therapeutic strategies in treating cancers.

Ion channels are transmembrane proteins allowing ions to cross biological membranes thereby generating electrical current and variation in membrane potential. Ion channels are classified in five main families, Potassium channels, Chloride channels, Sodium channels, Calcium channels that are selective for their specific ion, and non-selective ion channels that allow the passage of several species of ions. The functions attributed to ion channels were first confined to cell excitability and regulation of hydro-electrolytic balance. Interestingly, since the 1980's ion channels have also been described to be involved in controlling processes regulating cell behaviors such as proliferation,

migration, invasion and differentiation. In 1984, the pioneer work of DeCoursey describing the involvement of potassium channels in the proliferation of T lymphocytes (DeCoursey et al., 1984) opened new perspectives in studying the biology of ion channels. The first evidence for such functions of ion channels in cancer cells emerged in the late 1980's with studies describing that the pharmacological inhibition of ion channels reduces tumor cells proliferation (Yamashita et al., 1987; Lee et al., 1988; Pancrazio et al., 1989; Taylor and Simpson, 1992). Since then, the field has greatly expanded, and it has been shown that ion channels are involved in virtually all the hallmarks of cancers described by Hanahan et al. (Prevarskaya et al., 2010; Prevarskaya et al., 2018). Although the involvement of ion channels in cancer has been clearly described, how ion channels interact with cancer-related signaling pathways from a mechanistic point of view, and how the expression of ion channels is regulated in cancers is poorly understood. However, this issue has recently emerged in the literature and a growing number of reports tackle these questions. Cancer initiation and development is often associated to altered signaling pathway involved in cell and tissue homeostasis (Sanchez-Vega et al., 2018). Among these signaling pathways, the Wnt/β-catenin pathway emerged as the most associated pathway to ion channels in the literature. This review aims to summarize the current knowledge regarding the interplay between ion channels and the Wnt/β-catenin signaling pathway.

THE WNT SIGNALING PATHWAY

Since the first description of Wingless Int 1 (Wnt1) gene in virally induced breast tumors (Nusse and Varmus, 1982), the Wnt signaling pathway has been subjected to intense research, ranging from development to cancer. Its role in morphogenesis and adult stem cell renewal is closely linked to its involvement in cancer development (Nusse and Clevers, 2017). Mechanistically, Wnt signaling pathway can take mostly two directions: the canonical (Wnt/β-catenin), influencing cell-fate, proliferation, as well as self-renewal and the non-canonical (β-catenin-independent), Wnt/PCP, Wnt/Ca^{++}, Wnt/STOP, influencing planar polarity (Tree et al., 2002), Ca^{2+} homeostasis (Sheldahl et al., 2003), or protein stabilization (Davidson et al., 2009), respectively. Both are involved in cancer but canonical Wnt/β-catenin remains the most studied in a large variety of tissues.

In mammals, there are more than 19 Wnt ligands which are expressed in a tissue-specific manner. After their translation into proteins Wnt ligands are modified with attachment of a palmitoleic acid (palmitoylation) by the ER-resident acyl-transferase Porcupine (Kadowaki et al., 1996). This modification allows binding of Wnt ligand to the ER-transmembrane protein Evi/Wls, and therefore the transport to Golgi apparatus (Bänziger et al., 2006) assisted by the p24 protein (Buechling et al., 2011). Wnt ligands are either released by solubilisation (Mulligan et al., 2012), by exosomes (Gross et al., 2012), or in lipoproteins particles (Neumann et al., 2009) or linked to the plasma membrane. Importantly, after being secreted, Wnt ligands can form a gradient and act as directional growth factor, as it is the case in intestinal colonic crypts (Medema and Vermeulen, 2011)

Here we will focus on Wnt canonical pathway, which depends on β-catenin stability. In the absence of Wnt ligand (Wnt OFF state), cytosolic β-catenin is sequestrated by a destruction complex (DC) composed of the tumor suppressor Axin acting as scaffold protein, the tumor suppressor Adenomatous Polyposis Coli (APC), the glycogen synthase kinase 3β (GSK3β) and casein kinase 1α (CK1α). Inside this DC, β-catenin is bound to Axin and phosphorylated by the two constitutively active kinases GSK3β and CK1α at specific Serine and Threonine residues (Liu et al., 2002). Then, β-catenin is ubiquitylated by the F-box containing protein E3 ubiquitin ligase (β-TrCP) and addressed to the proteasome for degradation (Aberle et al., 1997; Latres et al., 1999).

Initiation of the Wnt canonical (β-catenin-dependent) pathway (Wnt ON state) is mediated by engagement of Frizzled (Fz) receptor and LRP5/6 co-receptor with Wnt ligands. Activation of the pathway leads to degradation complex relocation near Fz/LRP complexes, where LRP is phosphorylated by CK1α and GSK3β. The phosphorylation of LRP triggers Dishevelled (Dvl) protein polymerisation, thus inactivating the degradation complex (Metcalfe et al., 2010). Another study suggests a direct inhibition of GSK3β by the phosphorylated-LRP co-receptor (Stamos et al., 2014). Hence, β-catenin is not degraded, can accumulate in the cytoplasm and translocate into the nucleus. β-catenin then complexes with lymphoid enhanced factor (LEF) and T-cell factor (TCF) to act as a transcription factor activating target genes (Li et al., 2012). In the Wnt OFF state, TCF interacts with Groucho, preventing transcriptional activation of Wnt target genes (Cavallo et al., 1998). Interestingly, β-catenin plays a second role in maintaining epithelial junctions, by binding to the cytoplasmic tail of E-cadherin (Peifer et al., 1992). Thus, presence of epithelial junctions is a limiting factor for Wnt/β-catenin activity, and reciprocally (Huels et al., 2015).

The Wnt/β-catenin pathway is tightly regulated. For example, the two homologues E3 ubiquitin ligases ZNRF3/RNF43 can induce Fz receptor lysosomal degradation, thus inhibiting Wnt signaling. R-spondins are secreted proteins, interacting with their receptor LGR4-6, able to inhibit ZNRF3/RNF43 activity (de Lau et al., 2011). Furthermore, Hippo and Wnt pathway have been closely linked, since the Hippo transducer YAP/TAZ plays an integrative role inside the DC, allowing β-TrCP recruitment to the complex and subsequent β-catenin degradation (Azzolin et al., 2014). Others regulations are nicely reviewed by Zhan et al. (2017).

As mentioned above, Wnt canonical pathway is widely implicated in cancer diseases. The first mutations have been found in the APC gene, leading to Familiar Adenomatous Polyposis (Kinzler et al., 1991; Nishisho et al., 1991), and now found in the majority of colorectal cancers (Wood et al., 2007). Mutations of Axin1 are present in hepatocellular carcinoma (Satoh et al., 2000). Gain of function β-catenin mutations are reported in melanoma (Rubinfeld et al., 1997). Loss of function

mutations in Rnf43 or Znrf3 are found in pancreatic cancer (Wu et al., 2011) and adrenocortical carcinoma (Assié et al., 2014). Wnt signaling has also been implicated in cholangiosarcoma (Chan-On et al., 2013), leukemia (Wang et al., 2010) and in 50% of breast cancer cases (Lin et al., 2000).

WNT SIGNALING PATHWAY INTERPLAY WITH POTASSIUM CHANNELS

With more than 80 members, the potassium channel family is the widest group of ion channels. They participate in a multitude of biological processes from cell excitability to water and electrolyte homeostasis. They are especially essential in maintaining membrane resting potential. Increasing evidence point out the involvement of this family of ion channel in the physiopathology of cancers (Huang and Jan, 2014; Pardo and Stühmer, 2014). However, the molecular mechanisms sustaining potassium channels involvement in cancer are still unclear. This section reviews recent work that report the function of potassium channels in the Wnt/β-catenin pathway.

KCNQ1

The potassium channel KCNQ1, also called Kv7.1, belongs to the family of voltage-gated potassium channel. It is constituted by the assembly of four α subunits forming the pore of the channel. This channel can associate with ancillary subunits from the KCNE family which drastically change the channel features regarding the KCNE associated (Bendahhou et al., 2005; Jespersen et al., 2005). Association with KCNE1, shows a delay in KCNQ1 activation and in this case the complex KCNQ1/KCNE1 also called IKs, is essential to cardiac action potential repolarisation. With KCNE3 and KCNE2, KCNQ1 is no longer voltage-dependent and participates to transepithelial transport in nearly every epithelium. The effect of the last two members of the KCNE family on KCNQ1 are more discussed in the literature, however it appears that KCNE4 and KCNE5 mostly act as inhibitors of KCNQ1 activity (Abbott, 2014).

As aforementioned, KCNQ1 plays a key role in the physiology of epithelial transport. Transport mechanisms involve complex networks of ion channels and transporters, creating and dissipating ionic gradients required for the passage of water, electrolytes, nutrients or other substrates through the epithelium (Barrett and Keely, 2000). Thus, the Na^+/K^+ ATPase pump activity is the main force for transepithelial transport allowing Na^+ output against K^+ entry into the cell. In this context, K^+ channels provide two main functions: the maintenance of membrane potential and the basolateral recycling of K^+ that guarantees the maintenance of electrochemical gradients. In the colon, KCNQ1, associated with its regulatory subunit KCNE3, plays a fundamental role in Leiberkühn's crypts homeostasis by maintaining the electrical driving force required for apical secretion of Cl^- and water, and Na^+ absorption (Preston et al., 2010; Rapetti-Mauss et al., 2013).

Recently, studies found an unexpected link between KCNQ1 and epithelial cancers. In a first study, using mouse models, the authors have shown that the loss of KCNQ1 expression promotes the development of adenocarcinoma in the colon. They also showed that the low expression of KCNQ1 is associated with a poor survival prognosis in patients with Colorectal Cancer (CRC) showing hepatic metastases (Than et al., 2014). In line with these findings, another report has shown that in patients with stage II and III colon cancer, low expression of KCNQ1 is associated with poor survival prognosis. This channel therefore seems to be an interesting prognostic marker for the early stages of the disease as well as for the prediction of recurrences (den Uil et al., 2016). These studies also suggest that KCNQ1 acts as a tumor suppressor in CRC. Another study described the same function of KCNQ1 in Hepatocellular Carcinoma (HCC). They have shown using immunohistochemistry that HCC patients' samples show less KCNQ1 expression compared with normal liver tissues. Also, in patients with HCC, the high expression of KCNQ1 significantly correlates with a better patient's survival rate. Therefore, they reach the same conclusion as in CRC, KCNQ1 acts as a tumor suppressor in HCC (Fan et al., 2018).

It is clearly not trivial to explain the function of KCNQ1 as a tumor suppressor protein in epithelial cancers by its classical role in the physiology of epithelial transport. Therefore, could alternative functions of this channel underlie the observed correlations between KCNQ1 expression and cancers development? We and other have explored this issue to identify the molecular mechanism sustaining the tumor suppressor functions of KCNQ1 in CRC and HCC.

It appears that KCNQ1 expression is associated in CRC and HCC to epithelial cell plasticity (Rapetti-Mauss et al., 2017; Fan et al., 2018). Epithelial-to-Mesenchymal Transition (EMT) is a cellular process by which epithelial cells progressively and reversibly acquire mesenchymal features (Ye and Weinberg, 2015; Pastushenko and Blanpain, 2019). This process has largely been associated to cancer development and progression by allowing loss of epithelial structure and promoting cancer cell migration, invasion and drug resistance. In CRC, the expression of KCNQ1 in a panel of cell lines showing different degrees of EMT is associated with epithelial features. Conversely, the channel's expression is lowered in most mesenchymal cell lines. These data raise the hypothesis of a functional link between KCNQ1 and the cellular process of epithelial-to-mesenchymal transition in cancer cells. Interestingly, in CRC as well as in HCC, the rescue expression of KCNQ1 in cell lines that do not express this channel, restores the expression of epithelial markers such as E-cadherin, lowers the expression of mesenchymal markers such as N-cadherin and restores the ability of cell to form spheroids in 3D culture. Moreover, the molecular silencing of KCNQ1 in epithelial CRC cell line reduces the expression of E-cadherin, increases the expression of N-cadherin and impairs the ability to grow as a spheroid in 3D culture. Therefore, the expression of KCNQ1 seems to promote epithelial features.

An important event characterising EMT is the dislocation of adherens junctions (AJ) causing the loss of cohesion between adjacent cells and therefore disrupting the epithelial integrity. The

exploration of intestinal crypts by techniques coupling immunocytochemistry and in-situ immunisation (proximity ligation assay), provides data showing that KCNQ1 is directly associated with the β-catenin/E-cadherin complex in AJ in colonocytes. Interestingly, the same association between KCNQ1 and β-catenin has been established in hepatocyte cell lines. At the functional level, silencing of KCNQ1 expression in colon cell lines produces a disruption of the complex, as well as a loss of the integrity of epithelial architecture, which results in a decrease in epithelial electrical resistance. The study of the phosphorylation state of β-catenin in cell lines where KCNQ1 has been silenced by shRNA, indicates that KCNQ1 is necessary to retain β-catenin at the plasma membrane. Beside this observation it has been shown that pharmacological or molecular inhibition of KCNQ1 in CRC and in HCC, leads to a potentiation of the Wnt/β-catenin signaling pathway. Indeed, silencing of KCNQ1 in CRC induces the re-localisation of β-catenin in the cytosol and increases the expression of Wnt/β-catenin signaling pathway targets such as Cyclin D1 and C-jun. In HCC overexpression of KCNQ1 reduces Wnt target genes expression and Wnt activity using a Top/fop assay. These results indicate that KCNQ1 restricts the Wnt/β-catenin signaling pathway activity, stabilising β-catenin at the plasma membrane. These results reveal a new role for KCNQ1: in parallel with its function of transepithelial transport regulator, this channel plays a predominant role in maintaining the architectural integrity of the epithelium. These results shed new light on the correlation between the level of KCNQ1 expression and the clinical outcome of patients with CRC, since high levels of channel expression, together with the maintenance of adherens junctions guarantee the sequestration of β-catenin to the membrane, repression of the Wnt/β-catenin pathway, and finally reduces the commitment in the EMT process.

The stabilisation of the β-catenin/E-cadherin/KCNQ1 complex at the AJ constitutes an explanation to understand the role of KCNQ1 as a tumor suppressor in CRC and HCC. Would the latter only play an anchoring protein role for the β-catenin/E-cadherin complex, or is the KCNQ1-induced hyperpolarising potassium flow important in the stabilisation of the complex? In other words, do membrane potential variations influence β-catenin localisation and activity? The answer is provided by experiments in which CRC cells expressing KCNQ1 are experimentally depolarised by increasing the extracellular K^+ concentration 140mM K^+. This protocol causes a cytosolic accumulation of β-catenin, suggesting that the depolarisation induced by the silencing of the channel potentiates Wnt/β-catenin pathway. These results demonstrate the existence of a link between the electrical signature of the cancer cell and the signaling pathways that control cancer cells features. These data are consistent with a recent study that shows that the K-Ras/RAF/MAPK signaling pathway is directly controlled by membrane potential (Zhou et al., 2015).

There is only few information in the literature regarding how ion channel expression is regulated in the context of cancers. It has been observed that expression of KCNQ1 is decreased in both CRC and HCC. We have tempted to address this question and we reason that the link between KCNQ1 could be bidirectional. Is KCNQ1 expression repressed by Wnt activity? When the Wnt/β-catenin pathway is experimentally stimulated by pharmacological or molecular ways, we observed a decrease in KCNQ1 expression. We also identify that β-catenin as well as the TCF-4 transcription factor (member of the TCF/LEF family, which regulates the expression of the target genes of the Wnt pathway), bind directly to KCNQ1 promoter and cause the repression of its expression. These results thus reveal that KCNQ1 channel is a new target gene of Wnt pathway (Rapetti-Mauss et al., 2017).

By unveiling the bidirectional interaction between Wnt/β-catenin pathway and KCNQ1, these works reveal new functions of potassium channels in healthy tissue architecture, and their implication in the development of tumors. Thus, the fine understanding of these alternative functions could contribute to the characterisation of new markers, and innovative therapeutic targets. These data suggest that KCNQ1, by sequestering β-catenin at the AJ, restricts activation of Wnt signaling pathway and acts as a tumor suppressor in multiple epithelial cancers. The mechanism seems to be quite conserved suggesting a key role of KCNQ1 in epithelial homeostasis.

KCNH2

Another potassium channel has recently been associated to the Wnt/β-catenin pathway, KCNH2 also called hERG or Kv11.1 (Breuer et al., 2019). This channel is a voltage-gated potassium channel, which role in cancer cells has largely been studied especially by the groups of Arcangeli (Becchetti et al., 2019) and Soriani (Crottès et al., 2016). This channel is associated with β1-integrin when the channel is in its closed state, resulting in an increase of metastasis in breast cancer. Whereas, in the open state, the channel association with β1-integrin is decreased leading to a reduction in breast cancer metastasis (Becchetti et al., 2017).

A recent report described that an opener of hERG, the NS1643, reduces metastasis spread of breast tumor *in vivo* by attenuation of the Wnt/β catenin pathway signaling, suggesting that NS1643 mimics the dissociation between hERG and β1-integrin (Breuer et al., 2019). The authors observed that activating hERG channel stabilises β-catenin at the plasma membrane by inhibiting AKT (T308) and GSK3β (S9) phosphorylation. In this case the cytosolic fraction of β-catenin decreases favouring the association of β-catenin with E-cadherin. Therefore, NS1634 decreases Wnt pathway activity. This result suggests that the hyperproliferation induced by the opening of hERG reduces Wnt activity by retaining β-catenin at the plasma membrane. These data are consistent with the data from Arcangeli's lab showing that the open state of hERG lowers metastasis dissemination in breast cancer.

WNT SIGNALING PATHWAY INTERPLAY WITH CHLORIDE CHANNELS

Chloride channels regulate a variety of cell processes such as excitability and water and mucus secretion. Another important

aspect of these channels, especially in cancer, is their role in cell volume regulation and pH level regulation. As for the other ion channel families, different chloride channels have been described depending on their gating properties: calcium-activated, voltage-dependant, ligand-dependant. Although their role in cancer has been under study for the past years, their link with the Wnt/β-catenin pathway remains poorly investigated.

CFTR

Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) is widely expressed in the apical epithelial membranes. CFTR is an anion channel regulating fluid transport and pH levels. Mutations of CFTR such as ΔF508 result in Cystic Fibrosis (CF) causing a wide range of symptoms linked to a defect in fluid transport across the epithelial membranes. The pulmonary symptoms are well-described, but the earliest symptoms concern the digestive system. In addition, CF patients are at high risk of developing gastrointestinal cancers (Yamada et al., 2018). The study from Strubberg et al. links for the first time the loss of functional CFTR and gastrointestinal cancer through the increase of the proliferation rate of crypt cells by activating Wnt/β-catenin pathway (Strubberg et al., 2018). Indeed, the authors show that loss of functional CFTR leads to an increase of intracellular pH (pHi) levels in crypt cells as Cl^- and $HCO3^-$ secretion is impaired. In addition, it has been shown in *Drosophila* species that alkaline pHi (negatively charged phospholipids at the inner leaflet) facilitates Dishevelled's interaction with the Wnt receptor Frizzled by addressing Dishevelled to the negatively charged phospholipids through its DEP domain (Simons et al., 2009). Strubberg et al. show that the same mechanism is occurring in Cftr KO cells. The inhibition of CFTR activity alkalinizes pHi in the crypt epithelium, enhancing Dishevelled-2's (Dvl-2) association at the plasma membrane and promoting binding between Fz and Dvl-2 and therefore facilitating Wnt signaling. Confirming these results, active β-catenin and LEF1 levels were higher in Cftr KO mice crypts than WT. Furthermore, CFTR has been shown to be a direct target of Wnt signaling as it exhibits an intestine-specific enhancer element positively regulated by TCF4 (Paul et al., 2007). All in all, loss of CFTR facilitates Wnt signaling in the intestinal crypt epithelium, increasing tumorigenesis and underlying the basis of increased risk of gastrointestinal cancers in CF patients.

The main cancer risk for CF patients is gastrointestinal (GI) cancers (Neglia et al., 1995). However, as new therapeutic strategies aim to enhance CF patients' life expectancy, and since the link between loss of functional CFTR and Wnt/β-catenin pathway has been assessed in GI cancers, other epithelial cancers could occur in CF patients following the same biological process.

CLCN2

Adherens junctions and tight junctions ensure epithelium integrity, providing a physical barrier. Adherens junctions further establish the apical-basal cell polarity. The importance of the chloride channel ClCN2, also called ClC-2, in the regulation of tight junctions' composition in the small intestine has been recently uncovered (Nighot and Blikslager, 2012). As ClCN2 seemed to be an important actor of epithelial integrity and maintenance of the tight junctions, Jin *et al.* questioned its involvement in adherens junctions (AJ) and its interaction with β-catenin (Jin et al., 2018). β-catenin binds E-cadherin in the adherens junctions. If the AJ are disrupted, β-catenin is freed from the membrane-bound E-cadherin, accumulates in the cytosol and translocates to the nucleus where it interacts with TCF/LEF1, activating the Wnt target genes.

ClCN2 is expressed physiologically in the colonic epithelium. Absence of ClCN2 alters AJ integrity, inducing an increase of nuclear β-catenin. The increase of nuclear β-catenin is linked to an upregulation of Wnt target genes. Moreover, the loss of ClCN2 alters colonic crypt homeostasis, increases proliferation and reduces differentiation of colonic cells.

Therefore, the loss of ClCN2 in colonic epithelium leads to the disruption of adherens junction and subsequent activation of Wnt pathway, promoting tumorigenicity. The involvement of other ClCs in the regulation of junctions and their link with Wnt/β-catenin pathway has not yet been assessed. However, a study showed that absence of a regulatory protein of Calcium-activated chloride channels (CaCCs)—such as ANO1, also called TMEM16A (Sala-Rabanal et al., 2015; Sala-Rabanal et al., 2017)—CLCA1, induces an increase of β-catenin in the nucleus (Li et al., 2017). However, this study only focuses on the consequence of a knock-out of CLCA1 and not its link with CaCC channels nor its direct or indirect interaction with Wnt/β-catenin pathway. These points remain to be understood to truly consider CLCA1 as a tumor suppressor *via* inhibition of Wnt pathway.

WNT SIGNALING PATHWAY INTERPLAY WITH OTHER CHANNELS

TRP Channels

Transient receptor protein (TRP) channels are cationic channels that generate Na^+ and Ca^{2+} influxes in response to a great variety of stimuli (Venkatachalam and Montell, 2007). Therefore, TRP channels enable individual cells to sense changes in their local environment, translating this information into signals that can be interpreted at a short time scale such as membrane potential and Ca^{2+} homeostasis (Shapovalov et al., 2016). Several works have revealed a functional link between TRP channels and Wnt/β-catenin pathway in cancers.

TRPV4

In gastric cancers, the Calcium receptor (CasR) is overexpressed. Its overexpression contributes to tumor growth, metastasis formation, and poor prognosis. Mechanistically, CasR stimulates TRPV4 channels. The TRPV4-dependent Ca^{2+} influx increases AKT activation, which in turn phosphorylates β-catenin on its residue Ser675 and thus activating β-catenin downstream signaling (Xie et al., 2017).

TRPM4

TRPM4 channels are overexpressed in prostate and colorectal cancers where they participate to migration and invasion (Gao and Liao, 2019). In prostate cancer cells, invalidation of TRPM4 increases GSK3β activity, leading to β-catenin degradation. In LnCAP prostate cancer cells, TRPM4-associated Ca^{2+} influx stimulates AKT1, which in turn increases the inhibitory Ser9 phosphorylation of GSK3β and the total amount of β-catenin (Sagredo et al., 2018). In this study, Sagredo et al. suggest that the effect of TRPM4 on AKT1 is probably mediated by an alteration in the calcium/calmodulin-EGFR axis, linking TRPM4 activity with the observed effects in β-catenin-related signaling pathways.

TRPM8

A recent study driven in prostate and breast cancer cell lines has proposed that Wnt ligand-induced signaling involves TRPM8 channels. The authors propose a model in which Wnt ligands bind to both their receptors and TRPM8 channels, inducing a rapid decrease in $[Ca^{2+}]i$, followed by a store-operated calcium entry (SOCE). The SOCE in turn triggers calcium-dependent potassium channels (KCNN4) leading to a large membrane hyperpolarization. This study indeed suggests that control of membrane potential by Wnt ligands is an early event of the Wnt signaling pathway (Ashmore et al., 2019).

TRPC5

TRPC5 is overexpressed, together with ATP-binding cassette, subfamily 1-member1 (ABCB1) in 5-Fluorouracil (5-FU), in CRC cells. ABCB1 is a transport protein involved in chemoresistance by mediating efflux of cytotoxic drugs. Enforcing TrpC5 expression increases in a Ca^{2+}-dependent manner nuclear accumulation of β-catenin and subsequent upregulation of Wnt target genes including ABCB1. These results suggest that TRPC5 may reinforce chemoresistance by regulating transporters controlled by the Wnt/β-catenin pathway (Wang et al., 2015).

Purinergic Ionotropic Receptors

P2RX7

The ionotropic ATP receptor P2RX7, (P2X7) is overexpressed in osteocarcinoma and participates to several cancer hallmarks including proliferation, migration, invasion and EMT. At the molecular level, P2X7 stimulation by receptor agonist (bzATP) increases GSK3β phosphorylation and further accumulation of β-catenin in the nucleus. Indeed, Wnt/β-catenin activation by P2X7 stimulation depends on PI3K/AKT stimulation. Interestingly, pharmacological inhibition of P2X7 decreased pro-tumoral behavior of cancer cells (Zhang et al., 2019).

Acid Sensing Ion Channels

ASIC1a: Acid sensing ion channels (Asic) are proton-gated Na^+/Ca^{2+} channels involved in a wide range of physiological features including pain perception and behavior (Hanukoglu and Hanukoglu, 2016). Asic1a is up-regulated in liver cancer tissues and cells lines and is associated to poor prognosis. KO of Asic1a inhibits liver cancer growth *in vivo* and *in vitro*, an effect that is underlied by cell cycle arrest and increased apoptosis. Jin et al. observed that low extracellular pH

TABLE 1 | Role of ion channels in regulating Wnt pathway activity in cancers.

(C) Cancers Type	Models	Channels	Effect of channels expression/activity on Wnt pathway	Mechanism	Expression in cancers	References
Colorectal Cancer	Cell lines, Rat colonic crypts	KCNQ1	↓ Wnt activity Wnt target genes	Stabilization of β-cat. at the plasma membrane	Down	Rapetti-Mauss et al., 2017
Hepatocellular Carcinoma	Cell lines	KCNQ1	↓ Wnt activity	Stabilization of β-cat at the plasma membrane	Down	Fan et al., 2018,
Breast cancer	Mouse models, Cell lines	hERG	↓ Wnt activity (Channel opened)	Stabilization of β-cat at the plasma membrane	Up	Breuer et al., 2019
Colorectal cancer	Mouse intestine	CFTR	↓ Wnt activity Wnt target genes	Modulation of the intracellular pH	Down	Strubberg et al., 2018
Colorectal cancer	Cell lines; Mouse models	ClC-2	↓ Wnt activity	Stabilization of β-cat at the plasma membrane	Down	Jin et al., 2018
Gastric cancer	Human sample, mouse model, Cell lines	TRPV4	↑ Wnt activity	Ca^{2+} → AKT → β-cat accumulation	Up	Xie et al., 2017
Prostate cancer	Cell lines	TRPM4	↑ Wnt activity	Ca^{2+} → AKT → β-cat accumulation	Up	Sagredo et al., 2018
Prostate cancer Breast cancer	Cell lines	TRPM8	↑ Wnt activity	Wnt ligands binding → calcium	Up	Ashmore et al., 2019
Colorectal cancer	Cell lines	TRPC5	↑ Wnt activity	Ca^{2+} → AKT → β-cat accumulation	Up	Wang et al., 2015
osteosarcoma	Cell lines	P2X7	↑ Wnt activity	AKT →GSK3 β → β-cat accumulation	Up	Zhang et al., 2019
Liver cancer	Human sample, Cell lines	ASIC1a	↑ Wnt activity	Modulation of β-cat phosphorylation and accumulation	Up	Jin et al., 2017

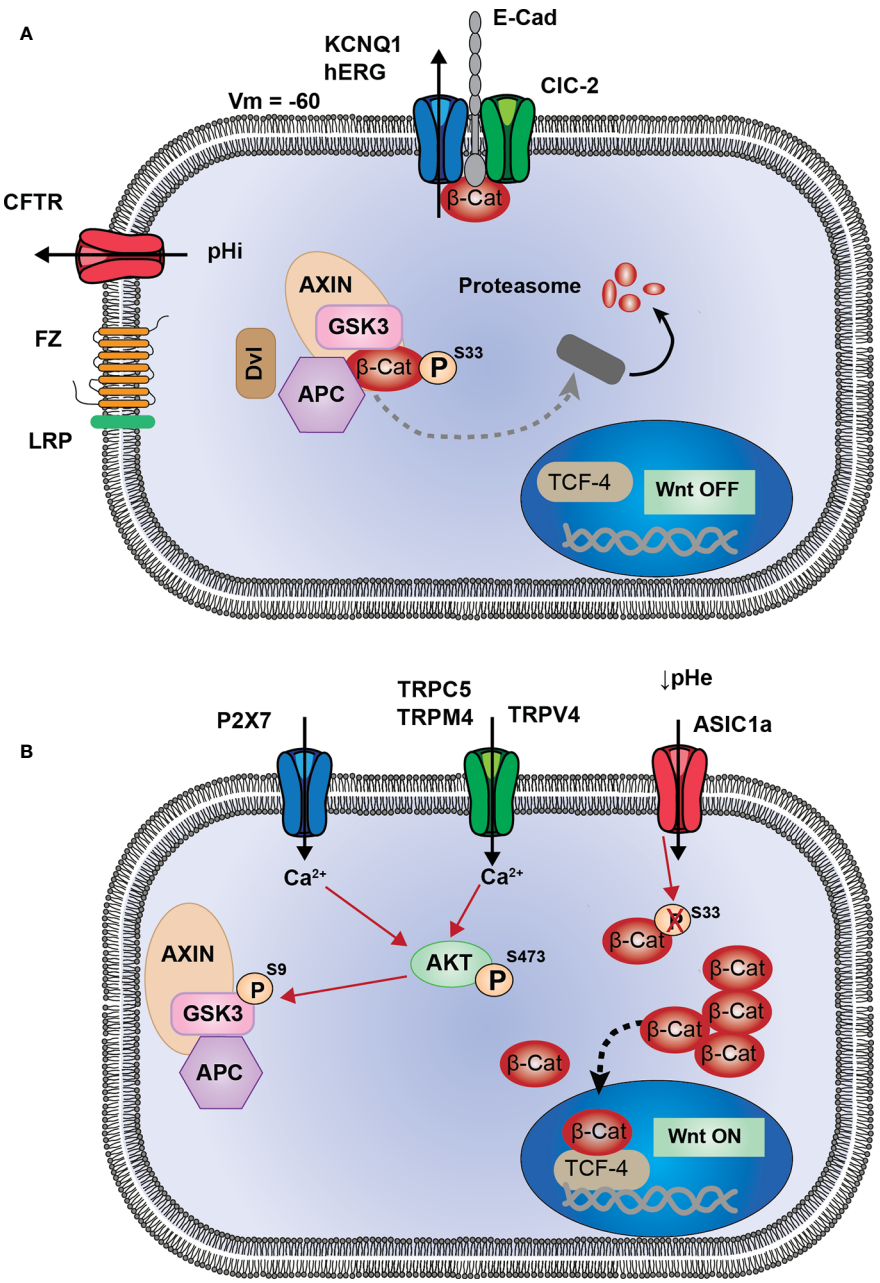


FIGURE 1 | Proposed model of interactions between ion channels and Wnt signaling. **(A)** KCNQ1 (or hERG) and ClC-2 are associated to E-cadherin and β-catenin at adherent junctions (AJ). Activity of these two channels maintain membrane potential hyperpolarized, which could be a favorable condition for AJ stability, and sequestration of β-catenin at the plasma membrane. CFTR activity maintains intracellular pH, preventing from pHi alkalinisation which induces Dishevelled-dependant inhibition of Destruction Complex (DC). These four channels participate in maintaining Wnt signaling in OFF-state, in which β-catenin is either degraded or sequestrated and cannot translocate into the nucleus to induce Wnt target genes. **(B)** Ca^{2+} entry through TRPC5, TRPM4, TRPV4 and P2X7 channels, induces AKT activation. Thus, AKT phosphorylates GSK3-β which becomes inhibited and subsequently allows the cytosolic accumulation of β-catenin. ASIC1a, by maintaining an acidic pH, decreases β-catenin phosphorylation and ubiquitination, preventing its degradation and inducing a cytosolic accumulation.

occurring in tumor microenvironment, increased Asic1a activity, which in turn decreases the Ser33 phosphorylation of β-catenin, leading to its accumulation in the nucleus, its combination with LEF/TCF promoting target gene transcription associated to liver cancer proliferation (Jin et al., 2017).

CONCLUSION AND FUTURE DIRECTIONS

In this review we have summarized the current work regarding the interplay between ion channels and the Wnt/β-catenin signaling pathway in cancers. The analysis of the literature

highlighted 10 ion channels associated to the Wnt pathway (**Table 1**). Among these channels four are associated to the repression of Wnt pathway (KCNQ1, hERG, CFTR, and ClCN-2, **Figure 1A**). The loss of activity or expression of these channels leads to an increase of Wnt activity and cancer progression. Supporting this, KCNQ1, CFTR and ClCN-2 have been described as tumor suppressors in CRC. Contrarily, six ion channels are overexpressed in cancers and promote the activity of Wnt pathway (TRPC5, TRPV4, TRPM4, TRPM8, P2RX7, and ASIC1a, **Figure 1B**).

Several conclusions can be drawn from this analysis of the literature. Firstly, the hyperpolarisation induced by the opening of potassium channel (background current from KCNQ1/KCNE3 in colonocytes or inducing the opening of hERG in breast cancer cells) leads to the restriction of Wnt activity by a similar mechanism, i.e. the sequestration of β-catenin at the plasma membrane and probably at the adherens junctions. Hyperpolarised plasma membrane seems to be important to prevent an over activation of the Wnt pathway and stabilise β-catenin at the plasma membrane. Therefore, the remaining question is: how can membrane potential act on β-catenin stability at the membrane? This is an outstanding question which will need more investigation to be answered. Secondly, regarding the Wnt-activating ion channels, the mechanism emerging from this analysis seems to mainly involve an increase in intracellular [Ca^{2+}] due to the overexpression or up regulation of cationic channels that mediate Ca^{2+} influx. The increase in intracellular [Ca^{2+}] shunts the canonical Wnt/β-catenin signaling cascade by activating AKT. AKT, in turn phosphorylates GSK3β on S9 causing its inhibition and finally the accumulation of β-catenin and the activation of Wnt pathway. Thirdly, as mentioned above, several ion channels are involved in the regulation of Wnt pathway, suggesting that interaction between them could exist. This is true especially regarding the case of KCNQ1, CFTR, and ClCN-2. It is interesting to note that KCNQ1 and ClCN-2 share the same mechanism to lower Wnt pathway activity. Both channels are associated to adherens junctions and contribute to stabilise β-catenin at the plasma membrane. This observation suggests a functional interaction between these channels which has not been investigated so far. Concerning KCNQ1 and CFTR, it is largely described that in colonic epithelium both channels are closely linked in the mechanism regulating the transepithelial transport of Cl^- and water. It seems credible that this functional link is conserved in the regulation of the Wnt pathway, both channels contributing to restrict its activity by two different, but complementary mechanisms, KCNQ1 controlling the stability of β-catenin at the plasma membrane and CFTR controlling the localization of Dvl. It will be interesting to study the effect of the simultaneous inhibition of both channels on Wnt activity.

Large research efforts have been made during the last 40 years to describe and understand the functions of ion channels in controlling cellular processes. Although the involvement of ion channels in processes participating in cancer development has largely been demonstrated, the precise mechanisms sustaining these abilities remain mainly unknown. Even if several functions of ion channels in signaling pathways can be explained by classical roles, such as regulation of cell volume or modulating the intracellular calcium concentration, other implication of this class of proteins are dictated by original mechanisms. Could ion channels be necessary in the regulation of classical signaling pathway and how? Can we insert ion channels in the notebook picture of signaling pathways beside tyrosine kinases receptors and G protein coupled receptors? Answering these questions are the arising challenges in the field of ion channels in cancer biology.

AUTHOR CONTRIBUTIONS

RR-M conceived the presented idea and supervised the project. RR-M, CB, BA, and OS wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Foundation ARC (grant PJA 20181207701), "La Fondation pour la Recherche Médicale"—FRM—(PhD studentship to CB) (n° ECO201906008972) and the Cancéropole PACA (grant n° 2018-13) and Université Côte d'Azur (CSI-2018).

REFERENCES

Abbott, G. W. (2014). Biology of the KCNQ1 Potassium Channel. *New J. Sci.* 2014, 26. doi: 10.1155/2014/237431

Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997). beta-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J.* 16, 3797–3804. doi: 10.1093/emboj/16.13.3797

Ashmore, J., Olsen, H., Sørensen, N., Thrasivoulou, C., and Ahmed, A. (2019). Wnts control membrane potential in mammalian cancer cells. *J. Physiol.* 597, 5899–5914. doi: 10.1113/JP278661

Assié, G., Letouzé, E., Fassnacht, M., Jouinot, A., Luscap, W., Barreau, O., et al. (2014). Integrated genomic characterization of adrenocortical carcinoma. *Nat. Genet.* 46, 607–612. doi: 10.1038/ng.2953

Azzolin, L., Panciera, T., Soligo, S., Enzo, E., Bicciato, S., Dupont, S., et al. (2014). YAP/TAZ incorporation in the β-catenin destruction complex orchestrates the Wnt response. *Cell* 158, 157–170. doi: 10.1016/j.cell.2014.06.013

Bänziger, C., Soldini, D., Schütt, C., Zipperlen, P., Hausmann, G., and Basler, K. (2006). Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signaling cells. *Cell* 125, 509–522. doi: 10.1016/j.cell.2006.02.049

Barrett, K. E., and Keely, S. J. (2000). Chloride secretion by the intestinal epithelium: molecular basis and regulatory aspects. *Annu. Rev. Physiol.* 62, 535–572. doi: 10.1146/annurev.physiol.62.1.535

Becchetti, A., Crescioli, S., Zanieri, F., Petroni, G., Mercatelli, R., Coppola, S., et al. (2017). The conformational state of hERG1 channels determines integrin association, downstream signaling, and cancer progression. *Sci. Signal.* 10 (473), eaaf3236. doi: 10.1126/scisignal.aaf3236

Becchetti, A., Petroni, G., and Arcangeli, A. (2019). Ion Channel Conformations Regulate Integrin-Dependent Signaling. *Trends Cell Biol.* 29, 298–307. doi: 10.1016/j.tcb.2018.12.005

Bendahhou, S., Marionneau, C., Haurogne, K., Larroque, M. M., Derand, R., Szuts, V., et al. (2005). In vitro molecular interactions and distribution of KCNE family with

KCNQ1 in the human heart. *Cardiovasc. Res.* 67, 529–538. doi: 10.1016/j.cardiores.2005.02.014

Breuer, E. K., Fukushiro-Lopes, D., Dalheim, A., Burnette, M., Zartman, J., Kaja, S., et al. (2019). Potassium channel activity controls breast cancer metastasis by affecting β-catenin signaling. *Cell Death Dis.* 10, 180. doi: 10.1038/s41419-019-1429-0

Buechling, T., Chaudhary, V., Spirohn, K., Weiss, M., and Boutros, M. (2011). p24 proteins are required for secretion of Wnt ligands. *EMBO Rep.* 12, 1265–1272. doi: 10.1038/embor.2011.212

Cavallo, R. A., Cox, R. T., Moline, M. M., Roose, J., Polevoy, G. A., Clevers, H., et al. (1998). Drosophila Tcf and Groucho interact to repress Wingless signalling activity. *Nature* 395, 604–608. doi: 10.1038/26982

Chan-On, W., Nairismägi, M. L., Ong, C. K., Lim, W. K., Dima, S., Pairojkul, C., et al. (2013). Exome sequencing identifies distinct mutational patterns in liver fluke-related and non-infection-related bile duct cancers. *Nat. Genet.* 45, 1474–1478. doi: 10.1038/ng.2806

Crottès, D., Rapetti-Mauss, R., Alcaraz-Perez, F., Tichet, M., Gariano, G., Martial, S., et al. (2016). SIGMAR1 Regulates Membrane Electrical Activity in Response to Extracellular Matrix Stimulation to Drive Cancer Cell Invasiveness. *Cancer Res.* 76, 607–618. doi: 10.1158/0008-5472.CAN-15-1465

Davidson, G., Shen, J., Huang, Y. L., Su, Y., Karaulanov, E., Bartscherer, K., et al. (2009). Cell cycle control of wnt receptor activation. *Dev. Cell.* 17, 788–799. doi: 10.1016/j.devcel.2009.11.006

de Lau, W., Barker, N., Low, T. Y., Koo, B. K., Li, V. S., Teunissen, H., et al. (2011). Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. *Nature* 476, 293–297. doi: 10.1038/nature10337

DeCoursey, T. E., Chandy, K. G., Gupta, S., and Cahalan, M. D. (1984). Voltage-gated K+ channels in human T lymphocytes: a role in mitogenesis? *Nature* 307, 465–468. doi: 10.1038/307465a0

den Uil, S. H., Coupé, V. M., Linnekamp, J. F., van den Broek, E., Goos, J. A., Delis-van Diemen, P. M., et al. (2016). Loss of KCNQ1 expression in stage II and stage III colon cancer is a strong prognostic factor for disease recurrence. *Br. J. Cancer* 115, 1565–1574. doi: 10.1038/bjc.2016.376

Fan, H., Zhang, M., and Liu, W. (2018). Hypermethylated KCNQ1 acts as a tumor suppressor in hepatocellular carcinoma. *Biochem. Biophys. Res. Commun.* 503, 3100–3107. doi: 10.1016/j.bbrc.2018.08.099

Gao, Y., and Liao, P. (2019). TRPM4 channel and cancer. *Cancer Lett.* 454, 66–69. doi: 10.1016/j.canlet.2019.04.012

Gross, J. C., Chaudhary, V., Bartscherer, K., and Boutros, M. (2012). Active Wnt proteins are secreted on exosomes. *Nat. Cell Biol.* 14, 1036–1045. doi: 10.1038/ncb2574

Hanukoglu, I., and Hanukoglu, A. (2016). Epithelial sodium channel (ENaC) family: Phylogeny, structure-function, tissue distribution, and associated inherited diseases. *Gene* 579, 95–132. doi: 10.1016/j.gene.2015.12.061

Huang, X., and Jan, L. Y. (2014). Targeting potassium channels in cancer. *J. Cell Biol.* 206, 151–162. doi: 10.1083/jcb.201404136

Huels, D. J., Ridgway, R. A., Radulescu, S., Leushacke, M., Campbell, A. D., Biswas, S., et al. (2015). E-cadherin can limit the transforming properties of activating β-catenin mutations. *EMBO J.* 34, 2321–2333. doi: 10.15252/embj.201591739

Jespersen, T., Grunnet, M., and Olesen, S. P. (2005). The KCNQ1 potassium channel: from gene to physiological function. *Physiol. (Bethesda)* 20, 408–416. doi: 10.1152/physiol.00031.2005

Jin, C., Yuan, F. L., Gu, Y. L., Li, X., Liu, M. F., Shen, X. M., et al. (2017). Over-expression of ASIC1a promotes proliferation via activation of the β-catenin/LEF-TCF axis and is associated with disease outcome in liver cancer. *Oncotarget* 8, 25977–25988. doi: 10.18632/oncotarget.10774

Jin, Y., Ibrahim, D., Magness, S. T., and Blikslager, A. T. (2018). Knockout of ClC-2 reveals critical functions of adherens junctions in colonic homeostasis and tumorigenicity. *Am. J. Physiol. Gastrointest. Liver Physiol.* 315, G966–G979. doi: 10.1152/ajpgi.00087.2018

Kadowaki, T., Wilder, E., Klingensmith, J., Zachary, K., and Perrimon, N. (1996). The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing. *Genes Dev.* 10, 3116–3128. doi: 10.1101/gad.10.24.3116

Kinzler, K. W., Nilbert, M. C., Su, L. K., Vogelstein, B., Bryan, T. M., Levy, D. B., et al. (1991). Identification of FAP locus genes from chromosome 5q21. *Science* 253, 661–665. doi: 10.1126/science.1651562

Latres, E., Chiaur, D. S., and Pagano, M. (1999). The human F box protein beta-Trcp associates with the Cul1/Skp1 complex and regulates the stability of beta-catenin. *Oncogene* 18, 849–854. doi: 10.1038/sj.onc.1202653

Lee, S. C., Deutsch, C., and Beck, W. T. (1988). Comparison of ion channels in multidrug-resistant and -sensitive human leukemic cells. *Proc. Natl. Acad. Sci. U. S. A.* 85, 2019–2023. doi: 10.1073/pnas.85.6.2019

Li, V. S., Ng, S. S., Boersema, P. J., Low, T. Y., Karthaus, W. R., Gerlach, J. P., et al. (2012). Wnt signaling through inhibition of β-catenin degradation in an intact Axin1 complex. *Cell* 149, 1245–1256. doi: 10.1016/j.cell.2012.05.002

Li, X., Hu, W., Zhou, J., Huang, Y., Peng, J., Yuan, Y., et al. (2017). CLCA1 suppresses colorectal cancer aggressiveness via inhibition of the Wnt/beta-catenin signaling pathway. *Cell Commun. Signal.* 15, 38. doi: 10.1186/s12964-017-0192-z

Lin, S. Y., Xia, W., Wang, J. C., Kwong, K. Y., Spohn, B., Wen, Y., et al. (2000). Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. *Proc. Natl. Acad. Sci. U. S. A.* 97, 4262–4266. doi: 10.1073/pnas.060025397

Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G. H., Tan, Y., et al. (2002). Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* 108, 837–847. doi: 10.1016/S0092-8674(02)00685-2

Medema, J. P., and Vermeulen, L. (2011). Microenvironmental regulation of stem cells in intestinal homeostasis and cancer. *Nature* 474, 318–326. doi: 10.1038/nature10212

Metcalfe, C., Mendoza-Topaz, C., Mieszczanek, J., and Bienz, M. (2010). Stability elements in the LRP6 cytoplasmic tail confer efficient signalling upon DIX-dependent polymerization. *J. Cell Sci.* 123, 1588–1599. doi: 10.1242/jcs.067546

Mulligan, K. A., Fuerer, C., Ching, W., Fish, M., Willert, K., and Nusse, R. (2012). Secreted Wingless-interacting molecule (Swim) promotes long-range signaling by maintaining Wingless solubility. *Proc. Natl. Acad. Sci. U. S. A.* 109, 370–377. doi: 10.1073/pnas.1119197109

Neglia, J. P., FitzSimmons, S. C., Maisonneuve, P., Schöni, M. H., Schöni-Affolter, F., Corey, M., et al. (1995). The risk of cancer among patients with cystic fibrosis. Cystic Fibrosis and Cancer Study Group. *N. Engl. J. Med.* 332, 494–499. doi: 10.1056/NEJM199502233320803

Neumann, S., Coudreuse, D. Y., van der Westhuyzen, D. R., Eckhardt, E. R., Korswagen, H. C., Schmitz, G., et al. (2009). Mammalian Wnt3a is released on lipoprotein particles. *Traffic* 10, 334–343. doi: 10.1111/j.1600-0854.2008.00872.x

Nighot, P. K., and Blikslager, A. T. (2012). Chloride channel ClC-2 modulates tight junction barrier function via intracellular trafficking of occludin. *Am. J. Physiol. Cell Physiol.* 302, C178–C187. doi: 10.1152/ajpcell.00072.2011

Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., et al. (1991). Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. *Science* 253, 665–669. doi: 10.1126/science.1651563

Nusse, R., and Clevers, H. (2017). Wnt/β-Catenin Signaling, Disease, and Emerging Therapeutic Modalities. *Cell* 169, 985–999. doi: 10.1016/j.cell.2017.05.016

Nusse, R., and Varmus, H. E. (1982). Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* 31, 99–109. doi: 10.1016/0092-8674(82)90409-3

Pancrazio, J. J., Viglione, M. P., Tabbara, I. A., and Kim, Y. I. (1989). Voltage-dependent ion channels in small-cell lung cancer cells. *Cancer Res.* 49, 5901–5906.

Pardo, L. A., and Stühmer, W. (2014). The roles of K(+) channels in cancer. *Nat. Rev. Cancer* 14, 39–48. doi: 10.1038/nrc3635

Pastushenko, I., and Blanpain, C. (2019). EMT Transition States during Tumor Progression and Metastasis. *Trends Cell Biol.* 29, 212–226. doi: 10.1016/j.tcb.2018.12.001

Paul, T., Li, S., Khurana, S., Leleiko, N. S., and Walsh, M. J. (2007). The epigenetic signature of CFTR expression is co-ordinated via chromatin acetylation through a complex intronic element. *Biochem. J.* 408, 317–326. doi: 10.1042/BJ20070282

Peifer, M., McCrea, P. D., Green, K. J., Wieschaus, E., and Gumbiner, B. M. (1992). The vertebrate adhesive junction proteins beta-catenin and plakoglobin and the Drosophila segment polarity gene armadillo form a multigene family with similar properties. *J. Cell Biol.* 118, 681–691. doi: 10.1083/jcb.118.3.681

Preston, P., Wartosch, L., Günzel, D., Fromm, M., Kongsuphol, P., Ousingsawat, J., et al. (2010). Disruption of the K+ channel beta-subunit KCNE3 reveals an important role in intestinal and tracheal Cl- transport. *J. Biol. Chem.* 285, 7165–7175. doi: 10.1074/jbc.M109.047829

Prevarskaya, N., Skryma, R., and Shuba, Y. (2010). Ion channels and the hallmarks of cancer. *Trends Mol. Med.* 16, 107–121. doi: 10.1016/j.molmed.2010.01.005

Prevarskaya, N., Skryma, R., and Shuba, Y. (2018). Ion Channels in Cancer: Are Cancer Hallmarks Oncochannelopathies? *Physiol. Rev.* 98, 559–621. doi: 10.1152/physrev.00044.2016

Rapetti-Mauss, R., O'Mahony, F., Sepulveda, F. V., Urbach, V., and Harvey, B. J. (2013). Oestrogen promotes KCNQ1 potassium channel endocytosis and postendocytic trafficking in colonic epithelium. *J. Physiol.* 591, 2813–2831. doi: 10.1113/jphysiol.2013.251678

Rapetti-Mauss, R., Bustos, V., Thomas, W., McBryan, J., Harvey, H., Lajczak, N., et al. (2017). Bidirectional KCNQ1:β-catenin interaction drives colorectal cancer cell differentiation. *Proc. Natl. Acad. Sci. U. S. A.* 114, 4159–4164. doi: 10.1073/pnas.1702913114

Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., and Polakis, P. (1997). Stabilization of beta-catenin by genetic defects in melanoma cell lines. *Science* 275, 1790–1792. doi: 10.1126/science.275.5307.1790

Sagredo, A. I., Sagredo, E. A., Cappelli, C., Báez, P., Andaur, R. E., Blanco, C., et al. (2018). TRPM4 regulates Akt/GSK3-β activity and enhances β-catenin signaling and cell proliferation in prostate cancer cells. *Mol. Oncol.* 12, 151–165. doi: 10.1002/1878-0261.12100

Sala-Rabanal, M., Yurtsever, Z., Nichols, C. G., and Brett, T. J. (2015). Secreted CLCA1 modulates TMEM16A to activate Ca(2+)-dependent chloride currents in human cells. *Elife17* 4, e05875. doi: 10.7554/eLife.05875

Sala-Rabanal, M., Yurtsever, Z., Berry, K. N., Nichols, C. G., and Brett, T. J. (2017). Modulation of TMEM16A channel activity by the von Willebrand factor type A (VWA) domain of the calcium-activated chloride channel regulator 1 (CLCA1). *J. Biol. Chem.* 292, 9164–9174. doi: 10.1074/jbc.M117.788232

Sanchez-Vega, F., Mina, M., Armenia, J., Chatila, W. K., Luna, A., La, K. C., et al. (2018). Oncogenic Signaling Pathways in The Cancer Genome Atlas. *Cell* 173, 321–337.e310. doi: 10.1016/j.cell.2018.03.035

Satoh, S., Daigo, Y., Furukawa, Y., Kato, T., Miwa, N., Nishiwaki, T., et al. (2000). AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. *Nat. Genet.* 24, 245–250. doi: 10.1038/73448

Shapovalov, G., Ritaine, A., Skryma, R., and Prevarskaya, N. (2016). Role of TRP ion channels in cancer and tumorigenesis. *Semin. Immunopathol.* 38, 357–369. doi: 10.1007/s00281-015-0525-1

Sheldahl, L. C., Slusarski, D. C., Pandur, P., Miller, J. R., Kühl, M., and Moon, R. T. (2003). Dishevelled activates Ca2+ flux, PKC, and CamKII in vertebrate embryos. *J. Cell Biol.* 161, 769–777. doi: 10.1083/jcb.200211094

Simons, M., Gault, W. J., Gotthardt, D., Rohatgi, R., Klein, T. J., Shao, Y., et al. (2009). Electrochemical cues regulate assembly of the Frizzled/Dishevelled complex at the plasma membrane during planar epithelial polarization. *Nat. Cell Biol.* 11, 286–294. doi: 10.1038/ncb1836

Stamos, J. L., Chu, M. L., Enos, M. D., Shah, N., and Weis, W. I. (2014). Structural basis of GSK-3 inhibition by N-terminal phosphorylation and by the Wnt receptor LRP6. *Elife* 3, e01998. doi: 10.7554/eLife.01998

Strubberg, A. M., Liu, J., Walker, N. M., Stefanski, C. D., MacLeod, R. J., Magness, S. T., et al. (2018). Cftr Modulates Wnt/β-Catenin Signaling and Stem Cell Proliferation in Murine Intestine. *Cell Mol. Gastroenterol. Hepatol.* 5, 253–271. doi: 10.1016/j.jcmgh.2017.11.013

Taylor, J. M., and Simpson, R. U. (1992). Inhibition of cancer cell growth by calcium channel antagonists in the athymic mouse. *Cancer Res.* 52, 2413–2418.

Than, B. L., Goos, J. A., Sarver, A. L., O'Sullivan, M. G., Rod, A., Starr, T. K., et al. (2014). The role of KCNQ1 in mouse and human gastrointestinal cancers. *Oncogene* 33, 3861–3868. doi: 10.1038/onc.2013.350

Tree, D. R., Shulman, J. M., Rousset, R., Scott, M. P., Gubb, D., and Axelrod, J. D. (2002). Prickle mediates feedback amplification to generate asymmetric planar cell polarity signaling. *Cell* 109, 371–381. doi: 10.1016/S0092-8674(02)00715-8

Venkatachalam, K., and Montell, C. (2007). TRP channels. *Annu. Rev. Biochem.* 76, 387–417. doi: 10.1146/annurev.biochem.75.103004.142819

Wang, Y., Krivtsov, A. V., Sinha, A. U., North, T. E., Goessling, W., Feng, Z., et al. (2010). The Wnt/beta-catenin pathway is required for the development of leukemia stem cells in AML. *Science* 327, 1650–1653. doi: 10.1126/science.1186624

Wang, T., Chen, Z., Zhu, Y., Pan, Q., Liu, Y., Qi, X., et al. (2015). Inhibition of transient receptor potential channel 5 reverses 5-Fluorouracil resistance in human colorectal cancer cells. *J. Biol. Chem.* 290, 448–456. doi: 10.1074/jbc.M114.590364

Wood, L. D., Parsons, D. W., Jones, S., Lin, J., Sjöblom, T., Leary, R. J., et al. (2007). The genomic landscapes of human breast and colorectal cancers. *Science* 318, 1108–1113. doi: 10.1126/science.1145720

Wu, J., Jiao, Y., Dal Molin, M., Maitra, A., de Wilde, R. F., Wood, L. D., et al. (2011). Whole-exome sequencing of neoplastic cysts of the pancreas reveals recurrent mutations in components of ubiquitin-dependent pathways. *Proc. Natl. Acad. Sci. U. S. A.* 108, 21188–21193. doi: 10.1073/pnas.1118046108

Xie, R., Xu, J., Xiao, Y., Wu, J., Wan, H., Tang, B., et al. (2017). Calcium Promotes Human Gastric Cancer via a Novel Coupling of Calcium-Sensing Receptor and TRPV4 Channel. *Cancer Res.* 77, 6499–6512. doi: 10.1158/0008-5472.CAN-17-0360

Yamada, A., Komaki, Y., Komaki, F., Micic, D., Zullow, S., and Sakuraba, A. (2018). Risk of gastrointestinal cancers in patients with cystic fibrosis: a systematic review and meta-analysis. *Lancet Oncol.* 19, 758–767. doi: 10.1016/S1470-2045(18)30188-8

Yamashita, N., Hamada, H., Tsuruo, T., and Ogata, E. (1987). Enhancement of voltage-gated Na+ channel current associated with multidrug resistance in human leukemia cells. *Cancer Res.* 47, 3736–3741.

Ye, X., and Weinberg, R. A. (2015). Epithelial-Mesenchymal Plasticity: A Central Regulator of Cancer Progression. *Trends Cell Biol.* 25, 675–686. doi: 10.1016/j.tcb.2015.07.012

Zhan, T., Rindtorff, N., and Boutros, M. (2017). Wnt signaling in cancer. *Oncogene* 36, 1461–1473. doi: 10.1038/onc.2016.304

Zhang, Y., Cheng, H., Li, W., Wu, H., and Yang, Y. (2019). Highly-expressed P2X7 receptor promotes growth and metastasis of human HOS/MNNG osteosarcoma cells via PI3K/Akt/GSK3β/β-catenin and mTOR/HIF1α/VEGF signaling. *Int. J. Cancer* 145, 1068–1082. doi: 10.1002/ijc.32207

Zhou, Y., Wong, C. O., Cho, K. J., van der Hoeven, D., Liang, H., Thakur, D. P., et al. (2015). SIGNAL TRANSDUCTION. Membrane potential modulates plasma membrane phospholipid dynamics and K-Ras signaling. *Science* 349, 873–876. doi: 10.1126/science.aaa5619

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 Rapetti-Mauss, Berenguier, Allegrini and Soriani. 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 Pharmacology

ORIGINAL RESEARCH
published: 02 October 2020
doi: 10.3389/fphar.2020.555047

Inhibitory Effect of Eslicarbazepine Acetate and S-Licarbazepine on $Na_v1.5$ Channels

Theresa K. Leslie[1], *Lotte Brückner*[1], *Sangeeta Chawla*[1,2] *and William J. Brackenbury*[1,2]*

[1] Department of Biology, University of York, York, United Kingdom, [2] York Biomedical Research Institute, University of York, York, United Kingdom

OPEN ACCESS

Edited by:
Sébastien Roger,
Université de Tours, France

Reviewed by:
Ricardo Gómez,
University of La Laguna, Spain
Carol Joy Milligan,
University of Melbourne, Australia

***Correspondence:**
William J. Brackenbury
william.brackenbury@york.ac.uk

Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: 23 April 2020
Accepted: 10 September 2020
Published: 02 October 2020

Citation:
Leslie TK, Brückner L, Chawla S and Brackenbury WJ (2020) Inhibitory Effect of Eslicarbazepine Acetate and S-Licarbazepine on $Na_v1.5$ Channels. Front. Pharmacol. 11:555047. doi: 10.3389/fphar.2020.555047

Eslicarbazepine acetate (ESL) is a dibenzazepine anticonvulsant approved as adjunctive treatment for partial-onset epileptic seizures. Following first pass hydrolysis of ESL, S-licarbazepine (S-Lic) represents around 95% of circulating active metabolites. S-Lic is the main enantiomer responsible for anticonvulsant activity and this is proposed to be through the blockade of voltage-gated Na^+ channels (VGSCs). ESL and S-Lic both have a voltage-dependent inhibitory effect on the Na^+ current in N1E-115 neuroblastoma cells expressing neuronal VGSC subtypes including $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.6$, and $Na_v1.7$. ESL has not been associated with cardiotoxicity in healthy volunteers, although a prolongation of the electrocardiographic PR interval has been observed, suggesting that ESL may also inhibit cardiac $Na_v1.5$ isoform. However, this has not previously been studied. Here, we investigated the electrophysiological effects of ESL and S-Lic on $Na_v1.5$ using whole-cell patch clamp recording. We interrogated two model systems: (1) MDA-MB-231 metastatic breast carcinoma cells, which endogenously express the "neonatal" $Na_v1.5$ splice variant, and (2) HEK-293 cells stably over-expressing the "adult" $Na_v1.5$ splice variant. We show that both ESL and S-Lic inhibit transient and persistent Na^+ current, hyperpolarise the voltage-dependence of fast inactivation, and slow the recovery from channel inactivation. These findings highlight, for the first time, the potent inhibitory effects of ESL and S-Lic on the $Na_v1.5$ isoform, suggesting a possible explanation for the prolonged PR interval observed in patients on ESL treatment. Given that numerous cancer cells have also been shown to express $Na_v1.5$, and that VGSCs potentiate invasion and metastasis, this study also paves the way for future investigations into ESL and S-Lic as potential invasion inhibitors.

Keywords: anticonvulsant, cancer, epilepsy, eslicarbazepine acetate, $Na_v1.5$, S-licarbazepine, voltage-gated Na^+ channel

INTRODUCTION

Eslicarbazepine acetate (ESL) is a member of the dibenzazepine anticonvulsant family of compounds which also includes oxcarbazepine and carbamazepine (Almeida and Soares-da-Silva, 2007). ESL has been approved by the European Medicines Agency and the United States Federal Drug Administration as an adjunctive treatment for partial-onset epileptic seizures (Sperling et al., 2015). ESL is administered orally and rapidly undergoes first pass hydrolysis to two stereoisomeric metabolites, R-licarbazepine and S-licarbazepine (S-Lic; also known as eslicarbazepine; **Figures 1A, B**) (Almeida et al., 2005; Almeida et al., 2008; Perucca et al., 2011). S-Lic represents around 95% of circulating active metabolites following first pass hydrolysis of ESL and is the enantiomer responsible for anticonvulsant activity (Potschka et al., 2014; Sierra-Paredes et al., 2014). S-Lic also has improved blood brain barrier penetration compared to R-licarbazepine (Alves et al., 2008). Although S-Lic has been shown to inhibit T type Ca^{2+} channels (Brady et al., 2011), its main activity is likely through blockade of voltage-gated Na^+ channels (VGSCs) (Hebeisen et al., 2015). ESL offers several clinical advantages over other older VGSC-inhibiting antiepileptic drugs, e.g. carbamazepine, phenytoin; it has a favourable safety profile (Brown and El-Mallakh, 2010; Hebeisen et al., 2015), reduced induction of hepatic cytochrome P450 enzymes (Galiana et al., 2017), low potential for drug-drug interactions (Falcao et al., 2012; Zaccara et al., 2015), and takes less time to reach a steady state plasma concentration (Bialer and Soares-da-Silva, 2012).

VGSCs are composed of a pore-forming α subunit in association with one or more auxiliary β subunits, the latter modulating channel gating and kinetics in addition to functioning as cell adhesion molecules (Catterall, 2014). There are nine α subunits ($Na_v1.1$-$Na_v1.9$), and four β subunits (β1-4) (Goldin et al., 2000; Brackenbury and Isom, 2011). In postnatal and adult CNS neurons, the predominant α subunits are the tetrodotoxin-sensitive $Na_v1.1$, $Na_v1.2$, and $Na_v1.6$ isoforms (Van Wart and Matthews, 2006) and it is therefore on these that the VGSC-inhibiting activity of ESL and S-Lic has been described. In the murine neuroblastoma N1E-115 cell line, which expresses $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.6$, and $Na_v1.7$, ESL and S-Lic both have a voltage-dependent inhibitory effect on the Na^+ current (Bonifacio et al., 2001; Hebeisen et al., 2015). In this cell model, S-Lic has no effect on the voltage-dependence of fast inactivation, but significantly hyperpolarises the voltage-dependence of slow inactivation (Hebeisen et al., 2015). S-Lic also has a lower affinity for VGSCs in the resting state than carbamazepine or oxcarbazepine, thus potentially improving its therapeutic window over first- and second-generation dibenzazepine compounds (Hebeisen et al., 2015). In acutely isolated murine hippocampal CA1 neurons, which express $Na_v1.1$, $Na_v1.2$ and $Na_v1.6$ (Westenbroek et al., 1989; Yu et al., 2006; Royeck et al., 2008), S-Lic significantly reduces the persistent Na^+ current, a very slow-inactivating component ~1% the size of the peak transient Na^+ current (Saint, 2008; Doeser et al., 2014). Moreover, in contrast to carbamazepine, this effect is maintained in the absence of β1 (Uebachs et al., 2010; Doeser et al., 2014).

In healthy volunteers, ESL has not been associated with cardiotoxicity and the QT interval remains unchanged on treatment (Vaz-Da-Silva et al., 2012). However, a prolongation of the PR interval has been observed (Vaz-Da-Silva et al., 2012), suggesting that caution should be exercised in patients with cardiac conduction abnormalities (Zaccara et al., 2015). Prolongation of the PR interval suggests that ESL may also inhibit the cardiac $Na_v1.5$ isoform, although this has not previously been studied. $Na_v1.5$ is not only responsible for the initial depolarisation of the cardiac action potential (George, 2005), but is also expressed in breast and colon carcinoma cells, where the persistent Na^+ current promotes invasion and

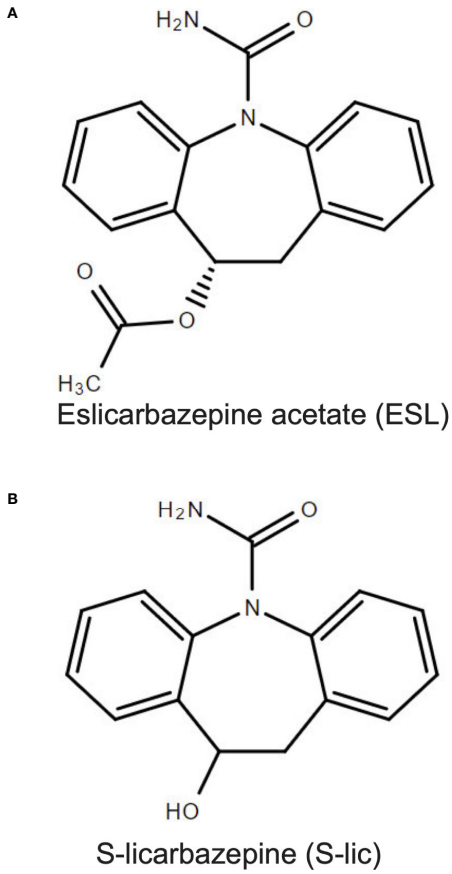


FIGURE 1 | Chemical structures of eslicarbazepine acetate and S-licarbazepine. **(A)** eslicarbazepine acetate; (9S)-2-carbamoyl-2-azatricyclo [9.4.0.0^{38}]pentadeca-1 (15),3,5,7,11,13-hexaen-9-yl acetate. **(B)** S-licarbazepine; (10R)-10-hydroxy-2-azatricyclo[9.4.0.$0^{3,8}$]pentadeca-1 (11),3,5,7,12,14-hexaene-2-carboxamide. Structures were drawn using Chemspider software.

Abbreviations: ESL, eslicarbazepine acetate; HEK-$Na_v1.5$, HEK-293 cells stably expressing $Na_v1.5$; I-V, current-voltage; k, slope factor; PSS, physiological saline solution; S-Lic, S-licarbazepin; T_p, time to peak current; τ_f, fast time constant of inactivation; τ_s, slow time constant of inactivation; τ_r, time constant of recovery from inactivation; VGSC, voltage-gated Na^+ channel; V_m, membrane potential; V_h, holding potential; V_{peak}, voltage at which current was maximal; V_{rev}, reversal potential; V_{thres}, threshold voltage for activation; $V_{1/2}$, half-activation voltage.

metastasis (Roger et al., 2003; Fraser et al., 2005; House et al., 2010; Nelson et al., 2015a). Inhibition of $Na_v1.5$ with phenytoin or ranolazine decreases tumor growth, invasion and metastasis (Yang et al., 2012; Driffort et al., 2014; Nelson et al., 2015b). Thus, it is of interest to specifically understand the effect of ESL on the $Na_v1.5$ isoform.

In the present study we investigated the electrophysiological effects of ESL and S-Lic on $Na_v1.5$ [1] endogenously expressed in the MDA-MB-231 metastatic breast carcinoma cell line, and [2] stably over-expressed in HEK-293 cells. We show that both ESL and S-Lic inhibit transient and persistent Na^+ current, hyperpolarise the voltage-dependence of fast inactivation, and slow the recovery from channel inactivation. These findings highlight, for the first time, the potent inhibitory effects of ESL and S-Lic on the $Na_v1.5$ isoform.

MATERIALS AND METHODS

Pharmacology

ESL (Tokyo Chemical Industry UK Ltd) was dissolved in DMSO to make a stock concentration of 67 mM. S-Lic (Tocris) was dissolved in DMSO to make a stock concentration of 300 mM. Both drugs were diluted to working concentrations of 100–300 μM in extracellular recording solution. The concentration of DMSO in the recording solution was 0.45% for ESL and 0.1% for S-Lic. Equal concentrations of DMSO were used in the control solutions. DMSO (0.45%) had no effect on the Na^+ current (**Supplementary Figure 1**).

Cell Culture

MDA-MB-231 cells and HEK-293 cells stably expressing $Na_v1.5$ (a gift from L. Isom, University of Michigan) were grown in Dulbecco's modified eagle medium supplemented with 5% FBS and 4 mM L-glutamine (Simon et al., 2020). Molecular identity of the MDA-MB-231 cells was confirmed by short tandem repeat analysis (Masters et al., 2001). Cells were confirmed as mycoplasma-free using the DAPI method (Uphoff et al., 1992). Cells were seeded onto glass coverslips 48 h before electrophysiological recording.

Electrophysiology

Plasma membrane Na^+ currents were recorded using the whole-cell patch clamp technique, using methods described previously (Yang et al., 2012; Nelson et al., 2015a). Patch pipettes made of borosilicate glass were pulled using a P-97 pipette puller (Sutter Instrument) and fire-polished to a resistance of 3–5 MΩ when filled with intracellular recording solution. The extracellular recording solution for MDA-MB-231 cells contained (in mM): 144 NaCl, 5.4 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 5.6 D-glucose, and 5 HEPES (adjusted to pH 7.2 with NaOH). For the extracellular recording solution for HEK-293 cells expressing $Na_v1.5$, the extracellular [Na^+] was reduced to account for the much larger Na^+ currents and contained (in mM): 60 NaCl, 84 Choline Cl, 5.4 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 5.6 D-glucose, and 5 HEPES (adjusted to pH 7.2 with NaOH). The intracellular recording solution contained (in mM): 5 NaCl, 145 CsCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 11 EGTA, (adjusted to pH 7.4 with CsOH) (Brackenbury and Djamgoz, 2006). Voltage clamp recordings were made at room temperature using a Multiclamp 700B or Axopatch 200B amplifier (Molecular Devices) compensating for series resistance by 40–60%. Currents were digitized using a Digidata interface (Molecular Devices), low pass filtered at 10 kHz, sampled at 50 kHz and analysed using pCLAMP 10.7 software (Molecular Devices). Leak current was subtracted using a P/6 protocol (Armstrong and Bezanilla, 1977). Extracellular recording solution ± drugs was applied to the recording bath at a rate of ~1.5 ml/min using a ValveLink 4-channel gravity perfusion controller (AutoMate Scientific). Each new solution was allowed to equilibrate in the bath for ~4 min following switching prior to recording at steady state.

Voltage Clamp Protocols

Cells were clamped at a holding potential of -120 mV or -80 mV for ≥250 ms, dependent on experiment (detailed in the Figure legends). Five main voltage clamp protocols were used, as follows:

1. To assess the effect of drug perfusion and wash-out on peak current in real time, a simple one-step protocol was used where cells were held at -120 mV or -80 mV for 250 ms and then depolarised to -10 mV for 50 ms.
2. To assess the voltage-dependence of activation, cells were held at -120 mV for 250 ms and then depolarised to test potentials in 10 mV steps between -120 mV and +30 mV for 50 ms. The voltage of activation was taken as the most negative voltage which induced a visible transient inward current.
3. To assess the voltage-dependence of steady-state inactivation, cells were held at -120 mV for 250 ms followed by prepulses for 250 ms in 10 mV steps between -120 mV and +30 mV and a test pulse to -10 mV for 50 ms.
4. To assess recovery from fast inactivation, cells were held at -120 mV for 250 ms, and then depolarised twice to 0 mV for 25 ms, returning to -120 mV for the following intervals between depolarisations (in ms): 1, 2, 3, 5, 7, 10, 15, 20, 30, 40, 50, 70, 100, 150, 200, 250, 350, 500. In each case, the second current was normalized to the initial current and plotted against the interval time.

Curve Fitting and Data Analysis

To study the voltage-dependence of activation, current-voltage (I-V) relationships were converted to conductance using the following equation:

$G = I/(V_m - V_{rev})$, where G is conductance, I is current, V_m is the membrane voltage and V_{rev} is the reversal potential for Na^+ derived from the Nernst equation. Given the different recording solutions used, V_{rev} for Na^+ was +85 mV for MDA-MB-231 cells and +63 mV for HEK-$Na_v1.5$ cells.

The voltage-dependence of conductance and availability were normalized and fitted to a Boltzmann equation:

$G = G_{max}/[1 + \exp((V_{1/2} - V_m)/k)]$, where G_{max} is the maximum conductance, $V_{1/2}$ is the voltage at which the channels are half

activated/inactivated, V_m is the membrane voltage and k is the slope factor.

Recovery from inactivation data ($I_t/I_{t=0}$) were normalized, plotted against recovery time (Δ;t) and fitted to a single exponential function:

$\tau = A_1 + A_2 \exp(-t/t_0)$, where A_1 and A_2 are the coefficients of decay of the time constant (τ), t is time and t_0 is a time constant describing the time dependence of τ.

The time course of inactivation was fitted to a double exponential function:

$I = A_f \exp(-t/\tau_f) + A_s \exp(-t/\tau_s) + C$, where A_f and A_s are maximal amplitudes of the slow and fast components of the current, τ_f and τ_s are the fast and slow decay time constants and C is the asymptote.

Statistical Analysis

Data are presented as mean and SEM unless stated otherwise. Statistical analysis was performed on the raw (non-normalized) data using GraphPad Prism 8.4.0. Pairwise statistical significance was determined with Student's paired *t*-tests. Multiple comparisons were made using ANOVA and Tukey post-hoc tests, unless stated otherwise. Results were considered significant at $P < 0.05$.

RESULTS

Effect of Eslicarbazepine Acetate and S-Licarbazepine on Transient and Persistent Na^+ Current

Several studies have clearly established the inhibition of neuronal VGSCs ($Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.6$, $Na_v1.7$ and $Na_v1.8$) by ESL and its active metabolite S-Lic (Bonifacio et al., 2001; Doeser et al., 2014; Hebeisen et al., 2015; Soares-da-Silva et al., 2015). Given that ESL prolongs the PR interval (Vaz-Da-Silva et al., 2012), potentially *via* inhibiting the cardiac $Na_v1.5$ isoform, together with the interest in inhibiting $Na_v1.5$ in carcinoma cells to reduce invasion and metastasis (Driffort et al., 2014; Martin et al., 2015; Nelson et al., 2015b; Elajnaf et al., 2018; Djamgoz et al., 2019), it is also relevant to evaluate the electrophysiological effects of ESL and S-Lic on this isoform. We therefore evaluated the effect of both compounds on $Na_v1.5$ current properties using whole-cell patch clamp recording, employing a two-pronged approach: (1) recording $Na_v1.5$ currents endogenously expressed in the MDA-MB-231 breast cancer cell line (Roger et al., 2003; Fraser et al., 2005; Brackenbury et al., 2007), and (2) recording from $Na_v1.5$ stably over-expressed in HEK-293 cells (HEK-$Na_v1.5$) (Patino et al., 2011).

Initially, we evaluated the effect of both compounds on the size of the peak Na^+ current in MDA-MB-231 cells. Na^+ currents were elicited by depolarising the membrane potential (V_m) to -10 mV from a holding potential (V_h) of -120 or -80 mV. Application of the prodrug ESL (300 μM) reversibly inhibited the transient Na^+ current by 49.6 ± 3.2% when the V_h was -120 mV ($P < 0.001$; n = 13; ANOVA + Tukey test; **Figures 2A, D**). When V_h was set to -80 mV, ESL (300 μM) reversibly inhibited the transient Na^+ current by 79.5 ± 4.5% ($P < 0.001$; n = 12; ANOVA + Tukey test; **Figures 2C, E**). We next assessed the effect of ESL in HEK-$Na_v1.5$ cells. Application of ESL (300 μM) inhibited $Na_v1.5$ current by 74.7 ± 4.3% when V_h was -120 mV ($P < 0.001$; n = 12; **Figures 2F, I**) and by 90.5 ± 2.8% when V_h was -80 mV ($P < 0.001$; n = 14; **Figures 2H, J**). However, the inhibition was only partially reversible ($P < 0.001$; n = 14; **Figures 2F, H–J**). Application of ESL at a lower concentration (100 μM) elicited a similar result (**Supplementary Figures 2A–J** and **Supplementary Table 1**). Together, these data suggest that ESL preferentially inhibited $Na_v1.5$ in the open or inactivated state, since the current inhibition was greater at more depolarised V_h.

We next tested the effect of the active metabolite S-Lic. S-Lic (300 μM) inhibited the transient Na^+ current in MDA-MB-231 cells by 44.4 ± 6.1% when the V_h was -120 mV ($P < 0.001$; n = 9; ANOVA + Tukey test; **Figures 3A, D**). When V_h was set to -80 mV, S-Lic (300 μM) inhibited the transient Na^+ current by 73.6 ± 4.1% ($P < 0.001$; n = 10; ANOVA + Tukey test; **Figures 3C, E**). However, the inhibition caused by S-Lic (300 μM) was only partially reversible ($P < 0.05$; n = 10; ANOVA + Tukey test; **Figures 3A, C–E**). In HEK-$Na_v1.5$ cells, S-Lic (300 μM) inhibited $Na_v1.5$ current by 46.4 ± 3.9% when V_h was -120 mV ($P < 0.001$; n = 13; ANOVA + Tukey test; **Figures 3F, I**) and by 74.0 ± 4.2% when V_h was -80 mV ($P < 0.001$; n = 12; ANOVA + Tukey test; **Figures 3H, J**). Furthermore, the inhibition in HEK-$Na_v1.5$ cells was not reversible over the duration of the experiment. Application of S-Lic at a lower concentration (100 μM) elicited a broadly similar result (**Supplementary Figures 3A–J** & **Supplementary Table 1**). Together, these data show that channel inhibition by S-Lic was also more effective at more depolarised V_h. However, unlike ESL, channel blockade by S-Lic persisted after washout, suggesting higher target binding affinity for the active metabolite and/or greater trapping of the active metabolite in the cytoplasm.

We also assessed the effect of both compounds on the persistent Na^+ current measured 20–25 ms after depolarisation to -10 from -120 mV. In MDA-MB-231 cells, ESL (300 μM) inhibited the persistent Na^+ current by 77 ± 34% although the reduction was not statistically significant ($P = 0.13$; n = 12; paired t test; **Figure 2B**, **Table 1**). In HEK-$Na_v1.5$ cells, ESL (300 μM) inhibited persistent current by 76 ± 10% ($P < 0.01$; n = 12; paired t test; **Figure 2G**, **Table 1**). S-Lic (300 μM) inhibited the persistent Na^+ current in MDA-MB-231 cells by 66 ± 16% ($P < 0.05$; n = 9; paired t test; **Figure 3B**, **Table 2**). In HEK-$Na_v1.5$ cells, S-Lic (300 μM) inhibited persistent current by 35 ± 16% ($P < 0.05$; n = 11; **Figure 3G**, **Table 2**). Application of both compounds at a lower concentration (100 μM) elicited a similar result (**Supplementary Table 1**). In summary, both ESL and S-Lic also inhibited the persistent Na^+ current.

Effect of Eslicarbazepine Acetate and S-Licarbazepine on Voltage Dependence of Activation and Inactivation

We next investigated the effect of ESL (300 μM) and S-Lic (300 μM) on the I-V relationship in MDA-MB-231 and HEK-$Na_v1.5$ cells. A V_h of -120 mV was used for subsequent analyses to ensure that the elicited currents were sufficiently large for

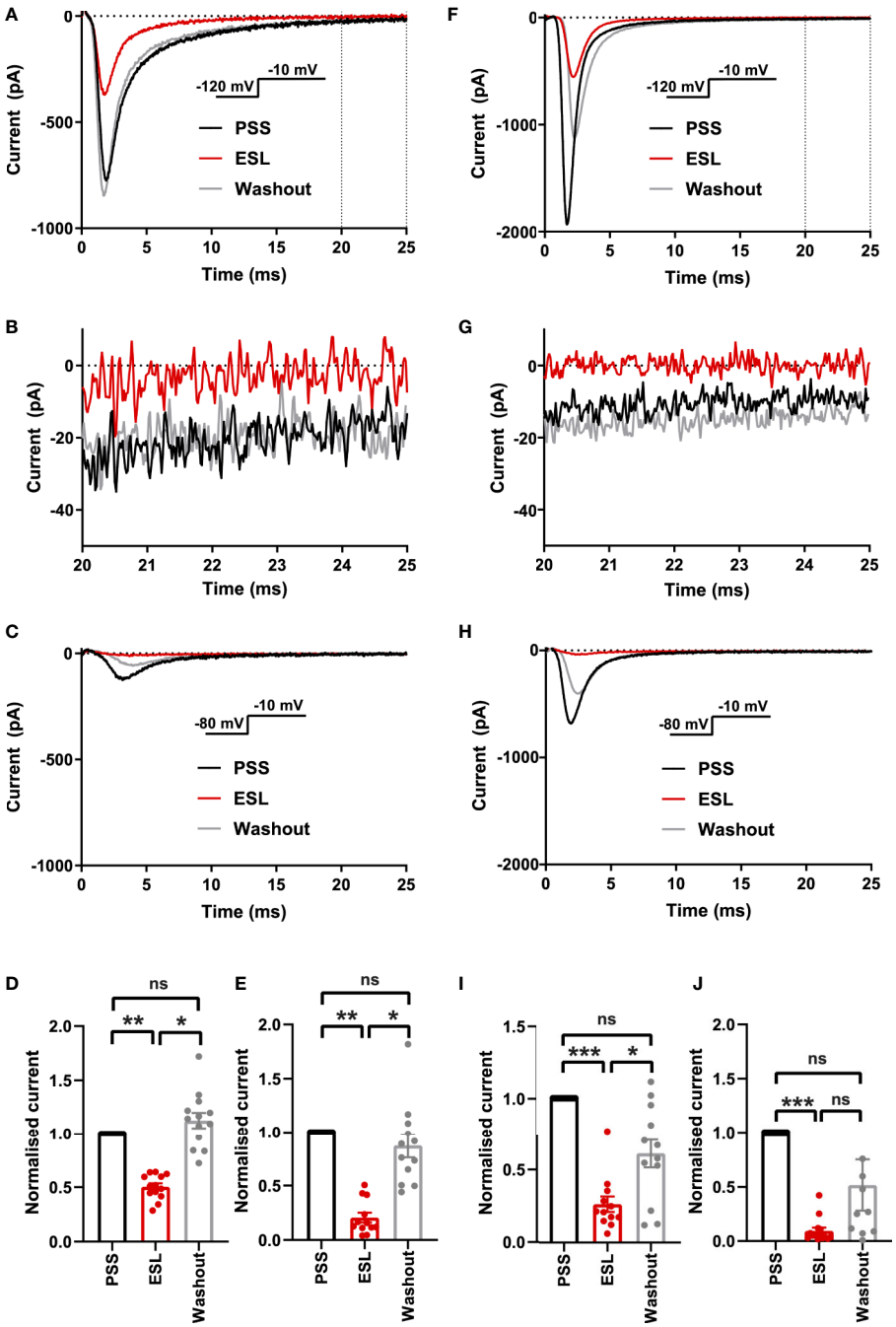


FIGURE 2 | Effect of eslicarbazepine acetate on $Na_v1.5$ currents. **(A)** Representative Na^+ currents in an MDA-MB-231 cell elicited by a depolarisation from -120 to -10 mV in physiological saline solution (PSS; black), eslicarbazepine acetate (ESL; 300 μM; red) and after washout (grey). Dotted vertical lines define the time period magnified in **(B)**. **(B)** Representative persistent Na^+ currents in an MDA-MB-231 cell elicited by a depolarisation from -120 to -10 mV. **(C)** Representative Na^+ currents in an MDA-MB-231 cell elicited by a depolarisation from -80 to -10 mV. **(D)** Normalized Na^+ currents in MDA-MB-231 cells elicited by a depolarisation from -120 to -10 mV. (E) Normalized Na^+ currents in MDA-MB-231 cells elicited by a depolarisation from -80 to -10 mV. **(F)** Representative Na^+ currents in a HEK-$Na_v1.5$ cell elicited by a depolarisation from -120 to -10 mV in PSS (black), ESL (300 μM; red) and after washout (grey). Dotted vertical lines define the time period magnified in **(G)**. **(G)** Representative persistent Na^+ currents in a HEK-$Na_v1.5$ cell elicited by a depolarisation from -120 to -10 mV. **(H)** Representative Na^+ currents in a HEK-$Na_v1.5$ cell elicited by a depolarisation from -80 to -10 mV. **(I)** Normalized Na^+ currents in HEK-$Na_v1.5$ cells elicited by a depolarisation from -120 to -10 mV. **(J)** Normalized Na^+ currents in HEK-$Na_v1.5$ cells elicited by a depolarisation from -80 to -10 mV. Results are mean + SEM. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; one-way ANOVA with Tukey tests (n = 12–14). NS, not significant.

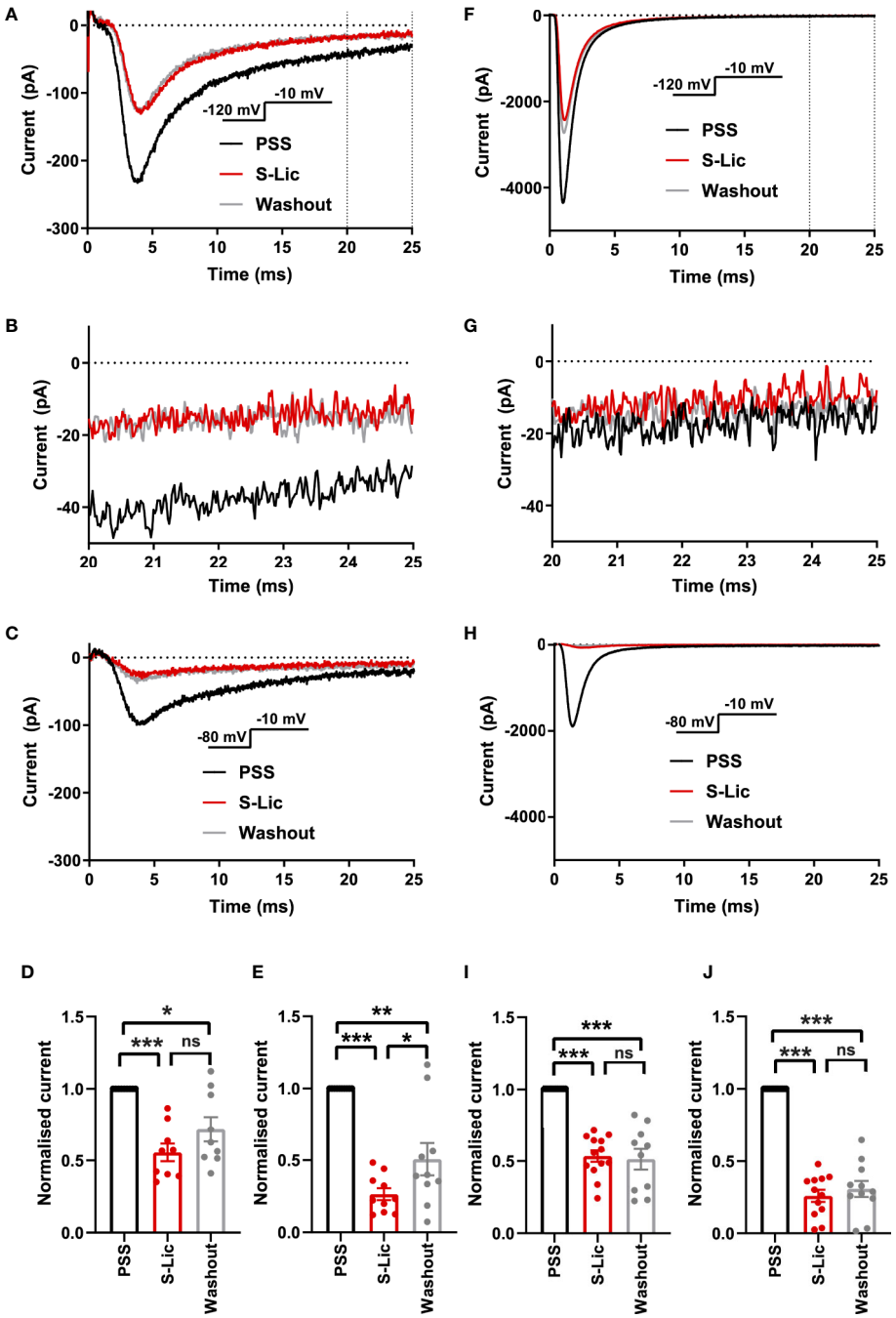


FIGURE 3 | Effect of S-licarbazepine on $Na_v1.5$ currents. **(A)** Representative Na^+ currents in an MDA-MB-231 cell elicited by a depolarisation from -120 to -10 mV in physiological saline solution (PSS; black), S-licarbazepine (S-Lic; 300 μM; red) and after washout (grey). Dotted vertical lines define the time period magnified in **(B)**. **(B)** Representative persistent Na^+ currents in an MDA-MB-231 cell elicited by a depolarisation from -120 to -10 mV. **(C)** Representative Na^+ currents in an MDA-MB-231 cell elicited by a depolarisation from -80 to -10 mV. **(D)** Normalized Na^+ currents in MDA-MB-231 cells elicited by a depolarisation from -120 to -10 mV. **(E)** Normalized Na^+ currents in MDA-MB-231 cells elicited by a depolarisation from -80 to -10 mV. **(F)** Representative Na^+ currents in a HEK-$Na_v1.5$ cell elicited by a depolarisation from -120 to -10 mV in PSS (black), S-Lic (300 μM; red) and after washout (grey). Dotted vertical lines define the time period magnified in **(G)**. **(G)** Representative persistent Na^+ currents in a HEK-$Na_v1.5$ cell elicited by a depolarisation from -120 to -10 mV. **(H)** Representative Na^+ currents in a HEK-$Na_v1.5$ cell elicited by a depolarisation from -80 to -10 mV. **(I)** Normalized Na^+ currents in HEK-$Na_v1.5$ cells elicited by a depolarisation from -120 to -10 mV. **(J)** Normalized Na^+ currents in HEK-$Na_v1.5$ cells elicited by a depolarisation from -80 to -10 mV. Results are mean + SEM. *P ≤ 0.05; ***P ≤ 0.001; one-way ANOVA with Tukey tests (n = 9-13). NS, not significant.

TABLE 1 | Effect of eslicarbazepine acetate (300 μM) on Na^+ current characteristics in MDA-MB-231 and HEK-Na_v1.5 cells.[1]

A. MDA-MB-231 cells

Parameter	*Control*	*ESL*	*P value*	*N*
V_{thres} (mV)	-45.7 ± 1.7	-45.0 ± 1.4	0.58	13
V_{peak} (mV)	3.1 ± 2.1	-3.9 ± 2.7	0.056	13
Activation V½ (mV)	-19.3 ± 1.4	-22.0 ± 1.5	0.095	12
Activation k (mV)	10.6 ± 0.7	9.3 ± 0.8	0.076	12
Inactivation V½ (mV)	-80.6 ± 0.7	-86.7 ± 1.2	< 0.001	13
Inactivation k (mV)	-4.8 ± 0.4	-7.4 ± 1.7	0.139	13
Peak current density at -10 mV (pA/pF)	-14.8 ± 3.9	-8.0 ± 2.5	< 0.001	13
Persistent current density at -10 mV (pA/pF)	-0.15 ± 0.05	-0.02 ± 0.07	0.13	12
T_p at -10 mV (ms)	2.1 ± 0.2	1.9 ± 0.2	< 0.01	13
τ_f at -10 mV (ms)	1.3 ± 0.1	1.3 ± 0.2	0.954	13
τ_s at -10 mV) (ms)	10.0 ± 2.3	6.9 ± 2.0	0.289	13
τ_r (ms)	6.0 ± 0.5	8.7 ± 0.7	< 0.05	10

B. HEK-Na_v1.5 cells

Parameter	*Control*	*ESL*	*P value*	*N*
V_{thres} (mV)	-55.0 ± 1.7	-54.0 ± 2.2	0.758	10
V_{peak} (mV)	-26.0 ± 2.2	-24.0 ± 4.3	0.591	10
Activation V½ (mV)	-39.4 ± 1.3	-44.2 ± 1.8	< 0.05	10
Activation k (mV)	5.3 ± 1.3	3.8 ± 0.7	0.361	10
Inactivation V½ (mV)	-78.2 ± 2.5	-88.3 ± 2.7	< 0.001	10
Inactivation k (mV)	-6.9 ± 0.4	-9.8 ± 0.7	< 0.001	10
Peak current density at -10 mV (pA/pF)	-154.4 ± 24.0	-33.1 ± 4.7	< 0.001	12
Persistent current density at -10 mV (pA/pF)	-0.61 ± 0.15	-0.12 ± 0.05	< 0.01	12
T_p at -10 mV (ms)	1.4 ± 0.2	1.9 ± 0.2	< 0.001	14
τ_f at -10 mV (ms)	0.9 ± 0.1	1.2 ± 0.1	< 0.001	12
τ_s at -10 mV (ms)	6.6 ± 0.8	20.8 ± 8.5	0.128	12
τ_r (ms)	4.5 ± 0.4	7.1 ± 0.6	< 0.001	10

[1]ESL, eslicarbazepine acetate (300 μM); V_{thres}, threshold voltage for activation; V_{peak}, voltage at which current was maximal; V½, half (in)activation voltage; k, slope factor for (in) activation; T_p, time to peak current; τ_f, fast time constant of inactivation; τ_s, slow time constant of inactivation; τ_r, time constant of recovery from inactivation. The holding potential was -120 mV. Results are mean ± SEM. Statistical comparisons were made with paired t-tests.

TABLE 2 | Effect of S-licarbazepine (300 μM) on Na^+ current characteristics in MDA-MB-231 and HEK-Na_v1.5 cells.[1]

A. MDA-MB-231 cells

Parameter	*Control*	*S-Lic*	*P value*	*N*
V_{thres} (mV)	-34.4 ± 2.0	-35.7 ± 2.0	0.603	7
V_{peak} (mV)	11.43 ± 4.4	10.0 ± 4.9	0.818	7
Activation V½ (mV)	-12.9 ± 1.3	-13.7 ± 1.4	0.371	7
Activation k (mV)	11.0 ± 0.5	11.9 ± 0.8	0.520	7
Inactivation V½ (mV)	-71.8 ± 2.5	-76.8 ± 2.2	< 0.05	7
Inactivation k (mV)	-6.8 ± 0.9	-6.0 ± 1.2	0.302	7
Peak current density at -10 mV (pA/pF)	-12.0 ± 3.1	-6.9 ± 2.5	< 0.001	9
Persistent current density at -10 mV (pA/pF)	-1.3 ± 0.4	-0.6 ± 0.2	< 0.05	9
T_p at -10 mV (ms)	4.5 ± 0.4	5.1 ± 0.7	0.103	9
τ_f at -10 mV (ms)	3.8 ± 1.1	3.2 ± 0.4	0.553	7
τ_s at -10 mV (ms)	25.7 ± 7.0	27.1 ± 12.0	0.920	7
τ_r (ms)	6.8 ± 0.4	13.5 ± 1.0	< 0.01	7

B. HEK-Na_v1.5 cells

Parameter	*Control*	*S-Lic*	*P value*	*N*
V_{thres} (mV)	-50.0 ± 1.9	-51.3 ± 3.5	0.598	9
V_{peak} (mV)	-18.0 ± 4.2	-30.0 ± 5.6	< 0.001	9
Activation V½ (mV)	-32.8 ± 3.1	-40.5 ± 3.4	< 0.01	9
Activation k (mV)	5.9 ± 0.9	4.5 ± 1.1	< 0.05	9
Inactivation V½ (mV)	-75.9 ± 2.6	-79.3 ± 4.1	0.116	9
Inactivation k (mV)	-6.5 ± 0.4	-8.1 ± 0.5	< 0.05	9
Peak current density at -10 mV (pA/pF)	-140.9 ± 26.8	-77.2 ± 17.0	< 0.001	13
Persistent current density at -10 mV (pA/pF)	-0.9 ± 0.2	-0.5 ± 0.2	< 0.05	11
T_p at -10 mV (ms)	1.8 ± 0.5	2.3 ± 0.6	< 0.01	13
τ_f at -10 mV (ms)	1.0 ± 0.04	1.3 ± 0.06	< 0.001	11
τ_s at -10 mV (ms)	6.3 ± 0.5	7.3 ± 0.5	< 0.05	11
τ_r (ms)	5.7 ± 0.7	8.0 ± 1.2	< 0.01	10

[1]S-Lic, S-licarbazepine (300 μM); V_{thres}, threshold voltage for activation; V_{peak}, voltage at which current was maximal; V½, half (in)activation voltage; k, slope factor for (in) activation; T_p, time to peak current; τ_f, fast time constant of inactivation; τ_s, slow time constant of inactivation; τ_r, time constant of recovery from inactivation. The holding potential was -120 mV. Results are mean ± SEM. Statistical comparisons were made with paired t-tests.

analysis of kinetics and voltage dependence, particularly for MDA-MB-231 cells, which display smaller peak Na^+ currents (**Tables 1**, **2**). Neither ESL nor S-Lic had any effect on the threshold voltage for activation (**Figures 4A–D**; **Tables 1**, **2**). ESL also had no effect on the voltage at current peak in either cell line (**Figures 4A–D**; **Tables 1**, **2**). Although S-Lic had no effect on voltage at current peak in MDA-MB-231 cells, it was significantly hyperpolarised in HEK-Na_v1.5 cells from -18.0 ± 4.2 to -30.0 ± 5.6 mV ($P < 0.001$; n = 9; paired t test; **Figures 4A–D**; **Tables 1**, **2**).

ESL had no significant effect on the half-activation voltage (V½) or slope factor (k) for activation in MDA-MB-231 cells (**Figure 5A**; **Table 1**). The activation k in HEK-Na_v1.5 cells was also unchanged but the activation V½ was significantly hyperpolarised by ESL from -39.4 ± 1.3 to -44.2 ± 1.8 mV ($P < 0.05$; n = 10; paired t test; **Figure 5B**; **Table 1**). S-Lic also had no significant effect on the activation V½ or k in MDA-MB-231 cells (**Figure 5C**; **Table 2**). However, the V½ of activation in HEK-Na_v1.5 cells was significantly hyperpolarised from -32.8 ± 3.1 to -40.5 ± 3.4 mV ($P < 0.01$; n = 9; paired t test; **Figure 5D**; **Table 2**) and k changed from 5.9 ± 0.9 to 4.5 ± 1.1 mV ($P < 0.05$; n = 9; paired t test; **Figure 5D**; **Table 2**).

As regards steady-state inactivation, in MDA-MB-231 cells, ESL significantly hyperpolarised the inactivation V½ from -80.6 ± 0.7 to -86.7 ± 1.2 mV ($P < 0.001$; n = 13; paired t test) without affecting inactivation k (**Figure 5A**; **Table 1**). ESL also hyperpolarised the inactivation V½ in HEK-Na_v1.5 cells from -78.2 ± 2.5 to -88.3 ± 2.7 mV ($P < 0.001$; n = 10; paired t test), and changed the inactivation k from -6.9 ± 0.4 to -9.8 ± 0.7 mV ($P < 0.001$; n = 10; paired t test; **Figure 5B**; **Table 1**). S-Lic also significantly hyperpolarised the inactivation V½ in MDA-MB-231 cells from -71.8 ± 2.5 to -76.8 ± 2.2 mV ($P < 0.05$; n = 7; paired t test) without affecting inactivation k (**Figure 5C**; **Table 2**). However, the inactivation V½ in HEK-Na_v1.5 cells was not significantly altered by S-Lic, although the inactivation k significantly changed from -6.5 ± 0.4 to -8.1 ± 0.5 mV ($P < 0.05$; n = 9; paired t test; **Figure 5D**; **Table 2**). In summary, both ESL and S-Lic affected various aspects of the voltage dependence characteristics of Na_v1.5 in MDA-MB-231 and HEK-Na_v1.5 cells, predominantly hyperpolarising the voltage dependence of inactivation.

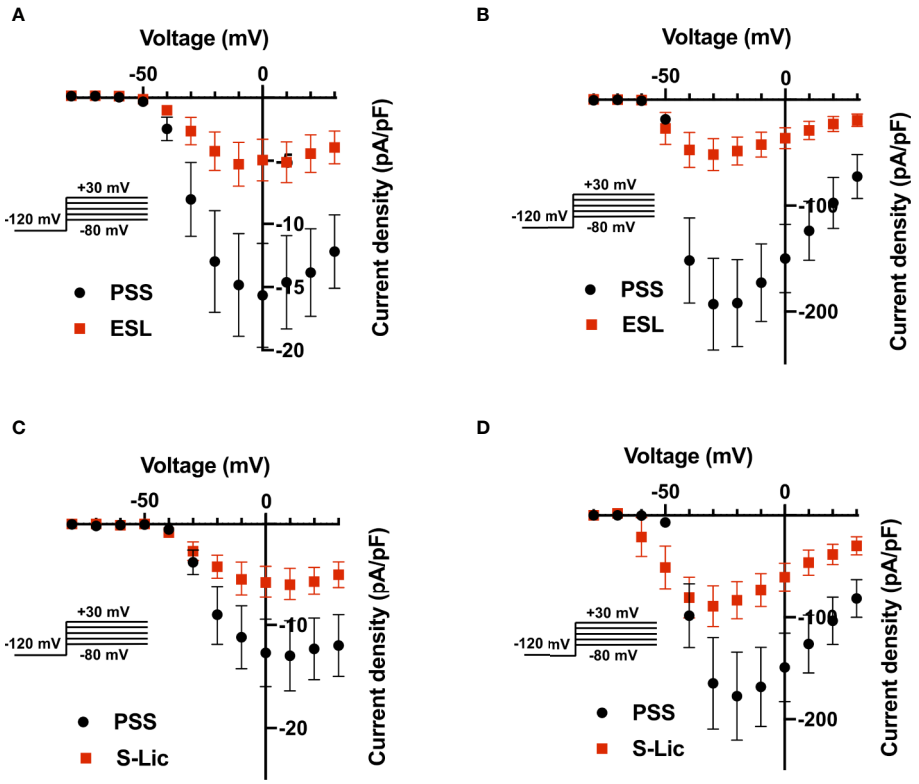


FIGURE 4 | Effect of eslicarbazepine acetate and S-licarbazepine on the current-voltage relationship. **(A)** Current-voltage (I–V) plots of Na^+ currents in MDA-MB-231 cells in physiological saline solution (PSS; black circles) and in eslicarbazepine acetate (ESL; 300 μM; red squares). **(B)** (I–V) plots of Na^+ currents in HEK-$Na_v1.5$ cells in PSS (black circles) and ESL (300 μM; red squares). **(C)** I–V plots of Na^+ currents in MDA-MB-231 cells in PSS (black circles) and S-licarbazepine (S-Lic; 300 μM; red squares). **(D)** I–V plots of Na^+ currents in HEK-$Na_v1.5$ cells in PSS (black circles) and S-Lic (300 μM; red squares). Currents were elicited using 10 mV depolarising steps from -80 to +30 mV for 30 ms, from a holding potential of -120 mV. Results are mean ± SEM (n = 7–13).

Effect of Eslicarbazepine Acetate and S-Licarbazepine on Activation and Inactivation Kinetics

We next studied the effect of both compounds on kinetics of activation and inactivation. In MDA-MB-231 cells, ESL (300 μM) significantly accelerated the time to peak current (T_p), upon depolarisation from -120 to -10 mV, from 2.1 ± 0.2 to 1.9 ± 0.2 ms ($P < 0.01$; n = 13; paired t test; **Table 1**). However, in HEK-$Na_v1.5$ cells, ESL significantly slowed T_p from 1.4 ± 0.2 to 1.5 ± 0.2 ms ($P < 0.001$; n = 14; paired t test; **Table 1**). S-Lic (300 μM) had no significant effect on T_p in MDA-MB-231 cells but significantly slowed T_p in HEK-$Na_v1.5$ cells from 1.8 ± 0.5 to 2.3 ± 0.6 ms ($P < 0.01$; n = 13; paired t test; **Table 2**).

To study effects on inactivation kinetics, the current decay following depolarisation from -120 to -10 mV was fitted to a double exponential function to derive fast and slow time constants of inactivation (τ_f and τ_s). Neither ESL nor S-Lic had any significant effect on τ_f or τ_s in MDA-MB-231 cells (**Tables 1**, **2**). However, in HEK-$Na_v1.5$ cells, ESL significantly slowed τ_f from 0.9 ± 0.1 to 1.2 ± 0.1 ms ($P < 0.001$; n = 12; paired t test; **Table 1**) and slowed τ_s from 6.6 ± 0.8 to 20.8 ± 8.5 ms, although this was not statistically significant. S-Lic significantly slowed τ_f from 1.0 ± 0.04 to 1.3 ± 0.06 ms ($P < 0.001$; n = 11; paired t test; **Table 2**) and τ_s from 6.3 ± 0.5 to 7.3 ± 0.5 ms ($P < 0.05$; n = 11; paired t test; **Table 2**). In summary, both ESL and S-Lic elicited various effects on kinetics in MDA-MB-231 and HEK-$Na_v1.5$ cells, predominantly slowing activation and inactivation.

Effect of Eslicarbazepine Acetate and S-Licarbazepine on Recovery From Fast Inactivation

To investigate the effect of ESL and S-Lic on channel recovery from fast inactivation, we subjected cells to two depolarisations from V_h of -120 to 0 mV, changing the interval between these in which the channels were held at -120 mV to facilitate recovery. Significance was determined by fitting a single exponential curve to the normalized current/time relationship and calculating the time constant (τ_r). In MDA-MB-231 cells, ESL (300 μM) significantly slowed τ_r from 6.0 ± 0.5 to 8.7 ± 0.7 ms ($P < 0.05$; n = 10; paired t test; **Figure 6A**, **Table 1**). Similarly, in HEK-$Na_v1.5$ cells, ESL significantly slowed τ_r from 4.5 ± 0.4 to 7.1 ± 0.6 ms ($P < 0.001$; n = 10; paired t test; **Figure 6B**, **Table 1**). S-Lic

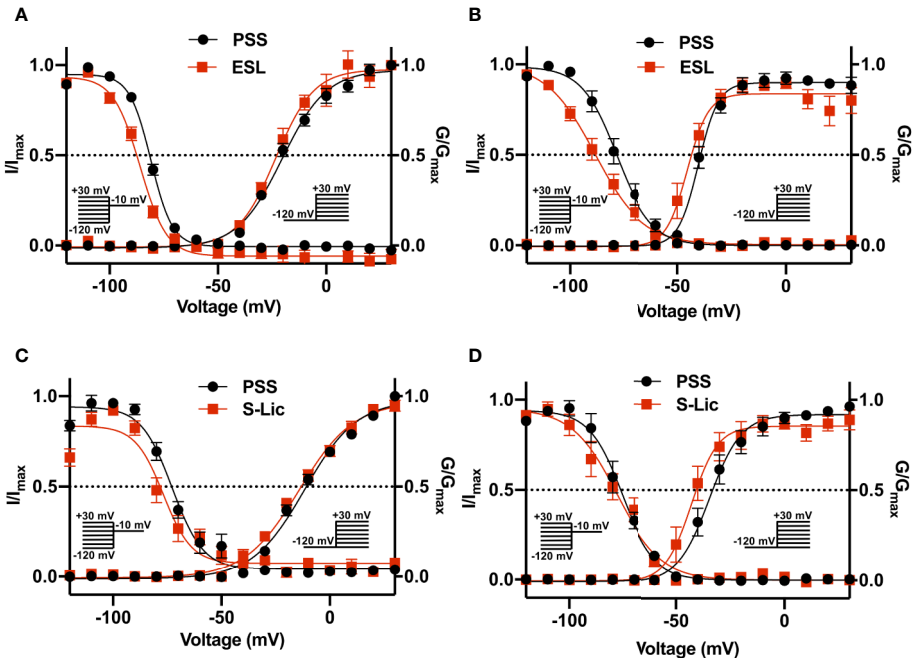


FIGURE 5 | Effect of eslicarbazepine acetate and S-licarbazepine on activation and steady-state inactivation. **(A)** Activation and steady-state inactivation in MDA-MB-231 cells in physiological saline solution (PSS; black circles) and in eslicarbazepine acetate (ESL; 300 μM; red squares). **(B)** Activation and steady-state inactivation in HEK-$Na_v1.5$ cells in PSS (black circles) and ESL (300 μM; red squares). **(C)** Activation and steady-state inactivation in MDA-MB-231 cells in PSS (black circles) and S-licarbazepine (S-Lic; 300 μM; red squares). **(D)** Activation and steady-state inactivation in HEK-$Na_v1.5$ cells in PSS (black circles) and S-Lic (300 μM; red squares). For activation, normalized conductance (G/G_{max}) was calculated from the current data and plotted as a function of voltage. For steady-state inactivation, normalized current (I/I_{max}), elicited by 50 ms test pulses at -10 mV following 250 ms conditioning voltage pulses between -120 and +30 mV, applied from a holding potential of -120 mV, was plotted as a function of the prepulse voltage. Results are mean ± SEM (n = 7–13). Activation and inactivation curves are fitted with Boltzmann functions.

(300 μM) also significantly slowed τ_r in MDA-MB-231 cells from 6.8 ± 0.4 to 13.5 ± 1.0 ms ($P < 0.01$; n = 7; paired t test; **Figure 6C**, **Table 2**). Finally, S-Lic also significantly slowed τ_r in HEK-$Na_v1.5$ cells from 5.7 ± 0.7 to 8.0 ± 1.2 ms ($P < 0.01$; n = 10; paired t test; **Figure 6D**, **Table 2**). In summary, both ESL and S-Lic slowed recovery from fast inactivation of $Na_v1.5$.

DISCUSSION

In this study, we have shown that ESL and its active metabolite S-Lic inhibit the transient and persistent components of Na^+ current carried by $Na_v1.5$. We show broadly similar effects in MDA-MB-231 cells, which express endogenous $Na_v1.5$ (Roger et al., 2003; Fraser et al., 2005; Brackenbury et al., 2007), and in HEK-293 cells over-expressing $Na_v1.5$. Notably, both compounds were more effective when V_h was set to -80 mV than at -120 mV, suggestive of depolarised state-dependent binding. In addition, the inhibitory effect of ESL was reversible whereas inhibition by S-Lic was less so. As regards voltage-dependence, both ESL and S-Lic shifted activation and steady-state inactivation curves, to varying extents in the two cell lines, in the direction of more negative voltages. ESL and S-Lic had various effects on activation and inactivation kinetics, generally slowing the rate of inactivation. Finally, recovery from fast inactivation of $Na_v1.5$ was significantly slowed by both ESL and S-Lic.

To our knowledge, this is the first time that the effects of ESL and S-Lic have specifically been tested on the $Na_v1.5$ isoform. A strength of this study is that both the prodrug (ESL) and the active metabolite (S-Lic) were tested using two independent cell lines, one endogenously expressing $Na_v1.5$, the other stably over-expressing $Na_v1.5$. MDA-MB-231 cells also express $Na_v1.7$, although this isoform is estimated to be responsible for only ~9% of the total VGSC current (Fraser et al., 2005; Brackenbury et al., 2007). MDA-MB-231 cells also express endogenous β1, β2, and β4 subunits (Chioni et al., 2009; Nelson et al., 2014; Bon et al., 2016). MDA-MB-231 cells predominantly express the developmentally regulated "neonatal" $Na_v1.5$ splice variant, which differs from the "adult" variant over-expressed in the HEK-$Na_v1.5$ cells by seven amino acids located in the extracellular linker between transmembrane segments 3 and 4 of domain 1 (Fraser et al., 2005; Brackenbury et al., 2007; Djamgoz et al., 2019). Notably, however, there were no consistent differences in effect of either ESL or S-Lic between the MDA-MB-231 and HEK-$Na_v1.5$ cells, suggesting that the neonatal vs. adult splicing event, and/or expression of

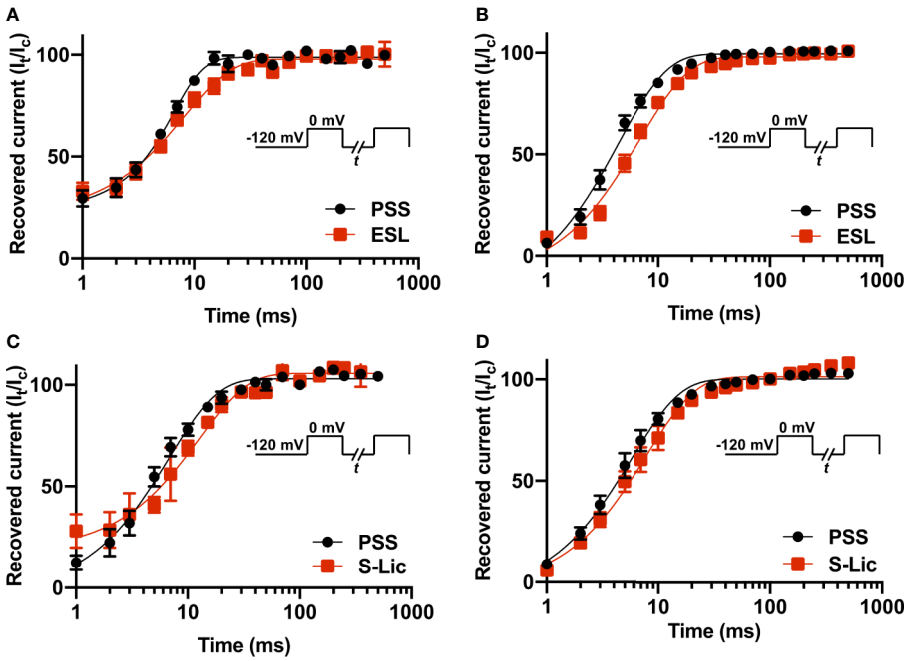


FIGURE 6 | Effect of eslicarbazepine acetate and S-licarbazepine on recovery from inactivation. **(A)** Recovery from inactivation in MDA-MB-231 cells in physiological saline solution (PSS; black circles) and in eslicarbazepine acetate (ESL; 300 μM; red squares). **(B)** Recovery from inactivation in HEK-$Na_v1.5$ cells in PSS (black circles) and ESL (300 μM; red squares). **(C)** Recovery from inactivation in MDA-MB-231 cells in PSS (black circles) and S-licarbazepine (S-Lic; 300 μM; red squares). **(D)** Recovery from inactivation in HEK-$Na_v1.5$ cells in PSS (black circles) and S-Lic (300 μM; red squares). The fraction recovered (I_t/I_c) was determined by a 25 ms pulse to 0 mV (I_c), followed by a recovery pulse to -120 mV for 1–500 ms, and a subsequent 25 ms test pulse to 0 mV (I_t), applied from a holding potential of -120 mV, and plotted as a function of the recovery interval. Data are fitted with single exponential functions which are statistically different between control and drug treatments in all cases. Results are mean ± SEM (n = 7–10).

endogenous β subunits, does not impact on sensitivity of $Na_v1.5$ to these compounds. This finding contrasts another report showing different sensitivity of the neonatal and adult $Na_v1.5$ splice variants to the amide local anaesthetics lidocaine and levobupivacaine (Elajnaf et al., 2018). Our findings suggest that the inhibitory effect of S-Lic on $Na_v1.5$ is less reversible than that of ESL. This may be explained by the differing chemical structures of the two molecules possibly enabling S-Lic to bind the target with higher affinity than ESL. Most VGSC-targeting anticonvulsants, including phenytoin, lamotrigine and carbamazepine, block the pore by binding *via* aromatic-aromatic interaction to a tyrosine and phenylalanine located in the S6 helix of domain 4 (Lipkind and Fozzard, 2010). However, S-Lic has been proposed to bind to a different site given that it was found to block the pore predominantly during slow inactivation (Hebeisen et al., 2015). Alternatively, the hydroxyl group present on S-Lic (but not ESL) may become deprotonated, potentially trapping it in the cytoplasm.

The findings presented here broadly agree with *in vitro* concentrations used elsewhere to study effects of ESL and S-Lic on Na^+ currents. For example, using a V_h of -80 mV, 300 μM ESL was shown to inhibit peak Na^+ current by 50% in N1E-115 neuroblastoma cells expressing $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.6$, and $Na_v1.7$ (Bonifacio et al., 2001). S-Lic (250 μM) also blocks peak Na^+ current by ~50% in the same cell line (Hebeisen et al., 2015). In addition, S-Lic (300 μM) reduces persistent Na^+ current by ~25% in acutely isolated murine hippocampal CA1 neurons expressing $Na_v1.1$, $Na_v1.2$, and $Na_v1.6$ (Westenbroek et al., 1989; Yu et al., 2006; Royeck et al., 2008; Doeser et al., 2014). Similar to the present study, ESL was shown to hyperpolarise the voltage-dependence of steady-state inactivation in N1E-115 cells (Bonifacio et al., 2001). On the other hand, similar to our finding in HEK-$Na_v1.5$ cells, S-Lic has no effect on steady-state inactivation in N1E-115 cells (Hebeisen et al., 2015). Again, in agreement with our own findings for $Na_v1.5$, S-Lic slows recovery from inactivation in N1E-115 cells (Hebeisen et al., 2015). These observations suggest that the sensitivity of $Na_v1.5$ to ESL and S-Lic is broadly similar to that reported for neuronal VGSCs. In support of this, $Na_v1.5$ shares the same conserved residues proposed for $Na_v1.2$ to interact with ESL (**Figure 7**) (Shaikh et al., 2014).

Notably, the concentrations used in this study are at or above those achieved in clinical use (e.g. ESL 1,200 mg once daily gives a peak plasma concentration of ~100 μM) (Hebeisen et al., 2015). However, it has been argued that the relatively high concentrations which have been previously tested *in vitro* are clinically relevant given that S-Lic has a high (50:1) lipid:water partition co-efficient and thus would be expected to reside predominantly in the tissue membrane fraction *in vivo* (Bialer and Soares-da-Silva, 2012). Our

```
SCN1A       ILENFSVATEESAEPLSEDDFEMFYEVWEKFDPDATQFMEFEKLSQFAAALEPPLNLPQP    1844
SCN2A       ILENFSVATEESAEPLSEDDFEMFYEVWEKFDPDATQFIEFAKLSDFADALDPPLLIAKP    1834
SCN3A       ILENFSVATEESAEPLSEDDFEMFYEVWEKFDPDATQFIEFSKLSDFAAALDPPLLIAKP    1829
SCN4A       ILENFNVATEESSEPLGEDDFEMFYETWEKFDPDATQFIAYSRLSDFVDTLQEPLRIAKP    1656
SCN5A       ILENFSVATEESTEPLSEDDFDMFYEIWEKFDPEATQFIEYSVLSDFADALSEPLRIAKP    1830
SCN8A       ILENFSVATEESADPLSEDDFETFYEIWEKFDPDATQFIEYCKLADFADALEHPLRVPKP    1824
SCN9A       ILENFSVATEESTEPLSEDDFEMFYEVWEKFDPDATQFIEFSKLSDFAAALDPPLLIAKP    1818
SCN10A      ILENFNVATEESTEPLSEDDFDMFYETWEKFDPEATQFITFSALSDFADTLSGPLRIPKP    1780
SCN11A      ILENFNTATEESEDPLGEDDFDIFYEVWEKFDPEATQFIKYSALSDFADALPEPLRVAKP    1662
            *****..***** :**.****: *** ******:****: :  *::*. :*  ** : :*

SCN1A       NKLQLIAMDLPMVSGDRIHCLDILFAFTKRVLGESGEMDALRIQMEERFMASNPSKVSYQ    1904
SCN2A       NKVQLIAMDLPMVSGDRIHCLDILFAFTKRVLGESGEMDALRIQMEERFMASNPSKVSYE    1894
SCN3A       NKVQLIAMDLPMVSGDRIHCLDILFAFTKRVLGESGEMDALRIQMEDRFMASNPSKVSYE    1889
SCN4A       NKIKLITLDLPMVPGDKIHCLDILFALTKEVLGDSGEMDALKQTMEEKFMAANPSKVSYE    1716
SCN5A       NQISLINMDLPMVSGDRIHCMDILFAFTKRVLGESGEMDALKIQMEEKFMAANPSKISYE    1890
SCN8A       NTIELIAMDLPMVSGDRIHCLDILFAFTKRVLGDSGELDILRQQMEERFVASNPSKVSYE    1884
SCN9A       NKVQLIAMDLPMVSGDRIHCLDILFAFTKRVLGESGEMDSLRSQMEERFMSANPSKVSYE    1878
SCN10A      NRNILIQMDLPLVPGDKIHCLDILFAFTKNVLGESGELDSLKANMEEKFMATNLSKSSYE    1840
SCN11A      NKYQFLVMDLPMVSEDRLHCMDILFAFTARVLGGSDGLDSMKAMMEEKFMEANPLKKLYE    1722
            *   :: :***:*  *::**:*****:* .*** *. :* ::  **::*: :*  *  *:
```

FIGURE 7 | Clustal alignment of amino acid sequences of $Na_v1.1$-$Na_v1.9$ (*SCN1A-SCN11A*). ESL was proposed previously (Shaikh et al., 2014) to interact with the highlighted amino acids in $Na_v1.2$. An alignment of $Na_v1.2$ [UniProtKB - Q99250 (SCN2A_HUMAN)] with $Na_v1.1$ [UniProtKB - P35498 (SCN1A_HUMAN)], $Na_v1.3$ [UniProtKB - Q9NY46 (SCN3A_HUMAN)], $Na_v1.4$ [UniProtKB - P35499 (SCN4A_HUMAN)], $Na_v1.5$ [UniProtKB - Q14524 (SCN5A_HUMAN)] $Na_v1.6$ [UniProtKB - Q9UQD0 (SCN8A_HUMAN)], $Na_v1.7$ [UniProtKB - Q15858 (SCN9A_HUMAN)], $Na_v1.8$ [UniProtKB - Q9Y5Y9 (SCN10A_HUMAN)], and $Na_v1.9$ [UniProtKB - Q9UI33 (SCN11A_HUMAN)] shows that the interacting amino acids highlighted in yellow are conserved between $Na_v1.2$ and $Na_v1.5$, along with most other isoforms. Asterisks indicate conserved residues. Colon indicates conservation between groups of strongly similar properties - scoring >0.5 in the Gonnet PAM 250 matrix. Period indicates conservation between groups of weakly similar properties - scoring ≤0.5 in the Gonnet PAM 250 matrix.

study suggests that a clinically relevant plasma concentration (100 μM) would inhibit peak and persistent $Na_v1.5$ currents. Future work investigating the dose-dependent effects of ESL and S-Lic would be useful to aid clinical judgements.

The data presented here raise several implications for clinicians. The observed inhibition of $Na_v1.5$ is worthy of note when considering cardiac function in patients receiving ESL (Zaccara et al., 2015). Although the QT interval remains unchanged for individuals on ESL treatment, prolongation of the PR interval has been observed (Vaz-Da-Silva et al., 2012). Further work is required to establish whether the basis for this PR prolongation is indeed *via* $Na_v1.5$ inhibition. In addition, it would be of interest to investigate the efficacy of ESL and S-Lic in the context of heritable arrhythmogenic mutations in *SCN5A*, as well as the possible involvement of the β subunits (Brackenbury and Isom, 2008; Uebachs et al., 2010; Doeser et al., 2014; Rivaud et al., 2020). The findings presented here are also relevant in the context of $Na_v1.5$ expression in carcinoma cells (Fraser et al., 2014). Given that cancer cells have a relatively depolarised V_m, it is likely that $Na_v1.5$ is mainly in the inactivated state with the persistent Na^+ current being functionally predominant (Yang and Brackenbury, 2013; Yang et al., 2020). Increasing evidence suggests that persistent Na^+ current carried by $Na_v1.5$ in cancer cells contributes to invasion and several studies have shown that other VGSC inhibitors reduce metastasis in preclinical models (Roger et al., 2003; Fraser et al., 2005; House et al., 2010; Yang et al., 2012; Driffort et al., 2014; Besson et al., 2015; Nelson et al., 2015a; Nelson et al., 2015b). Thus, use-dependent inhibition by ESL would ensure that channels in malignant cells are particularly targeted, raising the possibility that it could be used as an anti-metastatic agent (Martin et al., 2015). This study therefore paves the way for future investigations into ESL and S-Lic as potential invasion inhibitors.

DATA AVAILABILITY STATEMENT

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

TL, SC, and WB contributed to the conception and design of the work. TL, LB, and WB contributed to acquisition, analysis, and interpretation of data for the work. TL, SC, and WB contributed to drafting the work and revising it critically for important intellectual content. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by Cancer Research UK (A25922) and Breast Cancer Now (2015NovPhD572).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.555047/full#supplementary-material

REFERENCES

Almeida, L., and Soares-da-Silva, P. (2007). Eslicarbazepine acetate (BIA 2-093). *Neurotherapeutics.* 4 (1), 88–96. doi: 10.1016/j.nurt.2006.10.005

Almeida, L., Falcao, A., Maia, J., Mazur, D., Gellert, M., and Soares-da-Silva, P. (2005). Single-dose and steady-state pharmacokinetics of eslicarbazepine acetate (BIA 2-093) in healthy elderly and young subjects. *J. Clin. Pharmacol.* 45 (9), 1062–1066. doi: 10.1177/0091270005279364

Almeida, L., Minciu, I., Nunes, T., Butoianu, N., Falcão, A., Magureanu, S.-A., et al. (2008). Pharmacokinetics, Efficacy, and Tolerability of Eslicarbazepine Acetate in Children and Adolescents With Epilepsy. *J. Clin. Pharmacol.* 48 (8), 966–977. doi: 10.1177/0091270008319706

Alves, G., Figueiredo, I., Falcao, A., Castel-Branco, M., Caramona, M., and Soares-Da-Silva, P. (2008). Stereoselective disposition of S- and R-licarbazepine in mice. *Chirality.* 20 (6), 796–804. doi: 10.1002/chir.20546

Armstrong, C. M., and Bezanilla, F. (1977). Inactivation of the sodium channel. *II. Gating Curr. Exp. J. Gen. Physiol.* 70 (5), 567–590. doi: 10.1085/jgp.70.5.567

Besson, P., Driffort, V., Bon, E., Gradek, F., Chevalier, S., and Roger, S. (2015). How do voltage-gated sodium channels enhance migration and invasiveness in cancer cells? *Biochim. Biophys. Acta* 1848 (10 Pt B), 2493–2501. doi: 10.1016/j.bbamem.2015.04.013

Bialer, M., and Soares-da-Silva, P. (2012). Pharmacokinetics and drug interactions of eslicarbazepine acetate. *Epilepsia.* 53 (6), 935–946. doi: 10.1111/j.1528-1167.2012.03519.x

Bon, E., Driffort, V., Gradek, F., Martinez-Caceres, C., Anchelin, M., Pelegrin, P., et al. (2016). SCN4B acts as a metastasis-suppressor gene preventing hyperactivation of cell migration in breast cancer. *Nat. Commun.* 7, 13648. doi: 10.1038/ncomms13648

Bonifacio, M. J., Sheridan, R. D., Parada, A., Cunha, R. A., Patmore, L., and Soares-da-Silva, P. (2001). Interaction of the novel anticonvulsant, BIA 2-093, with voltage-gated sodium channels: comparison with carbamazepine. *Epilepsia.* 42 (5), 600–608. doi: 10.1046/j.1528-1157.2001.43600.x

Brackenbury, W. J., and Djamgoz, M. B. (2006). Activity-dependent regulation of voltage-gated Na^+ channel expression in Mat-LyLu rat prostate cancer cell line. *J. Physiol.* 573 (Pt 2), 343–356. doi: 10.1113/jphysiol.2006.106906

Brackenbury, W. J., and Isom, L. L. (2008). Voltage-gated Na+ channels: potential for beta subunits as therapeutic targets. *Expert Opin. Ther. Targets.* 12 (9), 1191–1203. doi: 10.1517/14728222.12.9.1191

Brackenbury, W. J., and Isom, L. L. (2011). Na Channel beta Subunits: Overachievers of the Ion Channel Family. *Front. Pharmacol.* 2:53. doi: 10.3389/fphar.2011.00053

Brackenbury, W. J., Chioni, A. M., Diss, J. K., and Djamgoz, M. B. (2007). The neonatal splice variant of Nav1.5 potentiates in vitro metastatic behaviour of MDA-MB-231 human breast cancer cells. *Breast Cancer Res. Treat.* 101 (2), 149–160. doi: 10.1007/s10549-006-9281-1

Brady, K., Hebeisen, S., Konrad, D., and Soares-da-Silva, P. (2011). The effects of eslicarbazepine, R-licarbazepine, oxcarbazepine and carbamazepine on ion transmission Cav3.2 channels. *Epilepsia.* 52, 260.

Brown, M. E., and El-Mallakh, R. S. (2010). Role of eslicarbazepine in the treatment of epilepsy in adult patients with partial-onset seizures. *Ther. Clin. Risk Manage.* 6, 103–109. doi: 10.2147/tcrm.s6382

Catterall, W. A. (2014). Structure and function of voltage-gated sodium channels at atomic resolution. *Exp. Physiol.* 99 (1), 35–51. doi: 10.1113/expphysiol.2013.071969

Chioni, A. M., Brackenbury, W. J., Calhoun, J. D., Isom, L. L., and Djamgoz, M. B. (2009). A novel adhesion molecule in human breast cancer cells: voltage-gated Na+ channel beta1 subunit. *Int. J. Biochem. Cell Biol.* 41 (5), 1216–1227. doi: 10.1016/j.biocel.2008.11.001

Djamgoz, M. B. A., Fraser, S. P., and Brackenbury, W. J. (2019). In Vivo Evidence for Voltage-Gated Sodium Channel Expression in Carcinomas and Potentiation of Metastasis. *Cancers (Basel).* 11 (11), 1–20. doi: 10.3390/cancers11111675

Doeser, A., Soares-da-Silva, P., Beck, H., and Uebachs, M. (2014). The effects of eslicarbazepine on persistent Na(+) current and the role of the Na(+) channel beta subunits. *Epilepsy Res.* 108 (2), 202–211. doi: 10.1016/j.eplepsyres.2013.11.022

Driffort, V., Gillet, L., Bon, E., Marionneau-Lambot, S., Oullier, T., Joulin, V., et al. (2014). Ranolazine inhibits NaV1.5-mediated breast cancer cell invasiveness and lung colonization. *Mol. Cancer.* 13 (1), 264. doi: 10.1186/1476-4598-13-264

Elajnaf, T., Baptista-Hon, D. T., and Hales, T. G. (2018). Potent Inactivation-Dependent Inhibition of Adult and Neonatal NaV1.5 Channels by Lidocaine and Levobupivacaine. *Anesth Analg.* 127 (3), 650–660. doi: 10.1213/ANE.0000000000003597

Falcao, A., Fuseau, E., Nunes, T., Almeida, L., and Soares-da-Silva, P. (2012). Pharmacokinetics, drug interactions and exposure-response relationship of eslicarbazepine acetate in adult patients with partial-onset seizures: population pharmacokinetic and pharmacokinetic/pharmacodynamic analyses. *CNS Drugs* 26 (1), 79–91. doi: 10.2165/11596290-000000000-00000

Fraser, S. P., Diss, J. K., Chioni, A. M., Mycielska, M. E., Pan, H., Yamaci, R. F., et al. (2005). Voltage-gated sodium channel expression and potentiation of human breast cancer metastasis. *Clin. Cancer Res.* 11 (15), 5381–5389. doi: 10.1158/1078-0432.CCR-05-0327

Fraser, S. P., Ozerlat-Gunduz, I., Brackenbury, W. J., Fitzgerald, E. M., Campbell, T. M., Coombes, R. C., et al. (2014). Regulation of voltage-gated sodium channel expression in cancer: hormones, growth factors and auto-regulation. *Philos. Trans. R. Soc. Lond B. Biol. Sci.* 369 (1638), 20130105. doi: 10.1098/rstb.2013.0105

Galiana, G. L., Gauthier, A. C., and Mattson, R. H. (2017). Eslicarbazepine Acetate: A New Improvement on a Classic Drug Family for the Treatment of Partial-Onset Seizures. *Drugs R. D.* 17 (3), 329–339. doi: 10.1007/s40268-017-0197-5

George, A. L.Jr. (2005). Inherited disorders of voltage-gated sodium channels. *J. Clin. Invest.* 115 (8), 1990–1999. doi: 10.1172/JCI25505

Goldin, A. L., Barchi, R. L., Caldwell, J. H., Hofmann, F., Howe, J. R., Hunter, J. C., et al. (2000). Nomenclature of voltage-gated sodium channels. *Neuron* 28, 365–368. doi: 10.1016/S0896-6273(00)00116-1

Hebeisen, S., Pires, N., Loureiro, A. I., Bonifacio, M. J., Palma, N., Whyment, A., et al. (2015). Eslicarbazepine and the enhancement of slow inactivation of voltage-gated sodium channels: a comparison with carbamazepine, oxcarbazepine and lacosamide. *Neuropharmacology* 89, 122–135. doi: 10.1016/j.neuropharm.2014.09.008

House, C. D., Vaske, C. J., Schwartz, A. M., Obias, V., Frank, B., Luu, T., et al. (2010). Voltage-gated Na+ channel SCN5A is a key regulator of a gene transcriptional network that controls colon cancer invasion. *Cancer Res.* 70 (17), 6957–6967. doi: 10.1158/0008-5472.CAN-10-1169

Lipkind, G. M., and Fozzard, H. A. (2010). Molecular model of anticonvulsant drug binding to the voltage-gated sodium channel inner pore. *Mol. Pharmacol.* 78 (4), 631–638. doi: 10.1124/mol.110.064683

Martin, F., Ufodiama, C., Watt, I., Bland, M., and Brackenbury, W. J. (2015). Therapeutic value of voltage-gated sodium channel inhibitors in breast, colorectal and prostate cancer: a systematic review. *Front. Pharmacol.* 6, 273. doi: 10.3389/fphar.2015.00273

Masters, J. R., Thomson, J. A., Daly-Burns, B., Reid, Y. A., Dirks, W. G., Packer, P., et al. (2001). Short tandem repeat profiling provides an international reference standard for human cell lines. *Proc. Natl. Acad. Sci. U. S. A.* 98 (14), 8012–8017. doi: 10.1073/pnas.121616198

Nelson, M., Millican-Slater, R., Forrest, L. C., and Brackenbury, W. J. (2014). The sodium channel beta1 subunit mediates outgrowth of neurite-like processes on breast cancer cells and promotes tumour growth and metastasis. *Int. J. Cancer.* 135 (10), 2338–2351. doi: 10.1002/ijc.28890

Nelson, M., Yang, M., Millican-Slater, R., and Brackenbury, W. J. (2015a). Nav1.5 regulates breast tumor growth and metastatic dissemination in vivo. *Oncotarget.* 6 (32), 32914–32929. doi: 10.18632/oncotarget.5441

Nelson, M., Yang, M., Dowle, A. A., Thomas, J. R., and Brackenbury, W. J. (2015b). The sodium channel-blocking antiepileptic drug phenytoin inhibits breast tumour growth and metastasis. *Mol. Cancer.* 14 (1), 13. doi: 10.1186/s12943-014-0277-x

Patino, G. A., Brackenbury, W. J., Bao, Y. Y., Lopez-Santiago, L. F., O'Malley, H. A., Chen, C. L., et al. (2011). Voltage-Gated Na+ Channel beta 1B: A Secreted Cell Adhesion Molecule Involved in Human Epilepsy. *J. Neurosci.* 31 (41), 14577–14591. doi: 10.1523/JNEUROSCI.0361-11.2011

Perucca, E., Elger, C., Halász, P., Falcão, A., Almeida, L., and Soares-da-Silva, P. (2011). Pharmacokinetics of eslicarbazepine acetate at steady-state in adults with partial-onset seizures. *Epilepsy Res.* 96 (1), 132–139. doi: 10.1016/j.eplepsyres.2011.05.013

Potschka, H., Soerensen, J., Pekcec, A., Loureiro, A., and Soares-da-Silva, P. (2014). Effect of eslicarbazepine acetate in the corneal kindling progression and the amygdala kindling model of temporal lobe epilepsy. *Epilepsy Res.* 108 (2), 212–222. doi: 10.1016/j.eplepsyres.2013.11.017

Rivaud, M. R., Delmar, M., and Remme, C. A. (2020). Heritable arrhythmia syndromes associated with abnormal cardiac sodium channel function: ionic and non-ionic mechanisms. *Cardiovasc. Res.* 116, 1557–1570. doi: 10.1093/cvr/cvaa082

Roger, S., Besson, P., and Le Guennec, J. Y. (2003). Involvement of a novel fast inward sodium current in the invasion capacity of a breast cancer cell line. *Biochim. Biophys. Acta* 1616 (2), 107–111. doi: 10.1016/j.bbamem.2003.07.001

Royeck, M., Horstmann, M. T., Remy, S., Reitze, M., Yaari, Y., and Beck, H. (2008). Role of axonal NaV1.6 sodium channels in action potential initiation of CA1 pyramidal neurons. *J. Neurophysiol.* 100 (4), 2361–2380. doi: 10.1152/jn.90332.2008

Saint, D. A. (2008). The cardiac persistent sodium current: an appealing therapeutic target? *Br. J. Pharmacol.* 153 (6), 1133–1142. doi: 10.1038/sj.bjp.0707492

Shaikh, S., Rizvi, S. M., Hameed, N., Biswas, D., Khan, M., Shakil, S., et al. (2014). Aptiom (eslicarbazepine acetate) as a dual inhibitor of beta-secretase and voltage-gated sodium channel: advancement in Alzheimer's disease-epilepsy linkage via an enzoinformatics study. *CNS Neurol. Disord. Drug Targets* 13 (7), 1258–1262. doi: 10.2174/18715273136661409171216 00

Sierra-Paredes, G., Loureiro, A. I., Wright, L. C., Sierra-Marcuño, G., and Soares-da-Silva, P. (2014). Effects of eslicarbazepine acetate on acute and chronic latrunculin A-induced seizures and extracellular amino acid levels in the mouse hippocampus. *BMC Neurosci.* 15 (1), 134. doi: 10.1186/s12868-014-0134-2

Simon, A., Yang, M., Marrison, J. L., James, A. D., Hunt, M. J., O'Toole, P. J., et al. (2020). Metastatic breast cancer cells induce altered microglial morphology and electrical excitability in vivo. *J. Neuroinflam.* 17 (1), 87. doi: 10.1186/s12974-020-01753-0

Soares-da-Silva, P., Pires, N., Bonifácio, M. J., Loureiro, A. I., Palma, N., and Wright, L. C. (2015). Eslicarbazepine acetate for the treatment of focal epilepsy: an update on its proposed mechanisms of action. *Pharmacol. Res. Perspect.* 3 (2), e00124. doi: 10.1002/prp2.124

Sperling, M. R., Abou-Khalil, B., Harvey, J., Rogin, J. B., Biraben, A., Galimberti, C. A., et al. (2015). Eslicarbazepine acetate as adjunctive therapy in patients with uncontrolled partial-onset seizures: Results of a phase III, double-blind, randomized, placebo-controlled trial. *Epilepsia* 56 (2), 244–253. doi: 10.1111/epi.12894

Uebachs, M., Opitz, T., Royeck, M., Dickhof, G., Horstmann, M. T., Isom, L. L., et al. (2010). Efficacy loss of the anticonvulsant carbamazepine in mice lacking sodium channel beta subunits via paradoxical effects on persistent sodium currents. *J. Neurosci.* 30 (25), 8489–8501. doi: 10.1523/JNEUROSCI.1534-10.2010

Uphoff, C. C., Gignac, S. M., and Drexler, H. G. (1992). Mycoplasma contamination in human leukemia cell lines. I. Comparison of various detection methods. *J. Immunol. Methods* 149 (1), 43–53. doi: 10.1016/s0022-1759(12)80047-0

Van Wart, A., and Matthews, G. (2006). Impaired firing and cell-specific compensation in neurons lacking nav1.6 sodium channels. *J. Neurosci.* 26 (27), 7172–7180. doi: 10.1523/JNEUROSCI.1101-06.2006

Vaz-Da-Silva, M., Nunes, T., Almeida, L., Gutierrez, M. J., Litwin, J. S., and Soares-Da-Silva, P. (2012). Evaluation of Eslicarbazepine acetate on cardiac repolarization in a thorough QT/QTc study. *J. Clin. Pharmacol.* 52 (2), 222–233. doi: 10.1177/0091270010391789

Westenbroek, R. E., Merrick, D. K., and Catterall, W. A. (1989). Differential subcellular localization of the RI and RII Na+ channel subtypes in central neurons. *Neuron.* 3 (6), 695–704. doi: 10.1016/0896-6273(89)90238-9

Yang, M., and Brackenbury, W. J. (2013). Membrane potential and cancer progression. *Front. Physiol.* 4, 185. doi: 10.3389/fphys.2013.00185

Yang, M., Kozminski, D. J., Wold, L. A., Modak, R., Calhoun, J. D., Isom, L. L., et al. (2012). Therapeutic potential for phenytoin: targeting Na(v)1.5 sodium channels to reduce migration and invasion in metastatic breast cancer. *Breast Cancer Res. Treat.* 134 (2), 603–615. doi: 10.1007/s10549-012-2102-9

Yang, M., James, A. D., Suman, R., Kasprowicz, R., Nelson, M., O'Toole, P. J., et al. (2020). Voltage-dependent activation of Rac1 by Nav 1.5 channels promotes cell migration. *J. Cell Physiol.* 235 (4), 3950–3972. doi: 10.1002/jcp.29290

Yu, F. H., Mantegazza, M., Westenbroek, R. E., Robbins, C. A., Kalume, F., Burton, K. A., et al. (2006). Reduced sodium current in GABAergic interneurons in a mouse model of severe myoclonic epilepsy in infancy. *Nat. Neurosci.* 9 (9), 1142–1149. doi: 10.1038/nn1754

Zaccara, G., Giovannelli, F., Cincotta, M., Carelli, A., and Verrotti, A. (2015). Clinical utility of eslicarbazepine: current evidence. *Drug Des. Dev. Ther.* 9, 781–789. doi: 10.2147/DDDT.S57409

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 Leslie, Brückner, Chawla and Brackenbury. 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 Pharmacology

REVIEW
published: 16 October 2020
doi: 10.3389/fphar.2020.568993

Ion Channel Signature in Healthy Pancreas and Pancreatic Ductal Adenocarcinoma

Julie Schnipper[1], *Isabelle Dhennin-Duthille*[1], *Ahmed Ahidouch*[1,2] *and Halima Ouadid-Ahidouch*[1]*

[1] Laboratory of Cellular and Molecular Physiology, UR-4667, University of Picardie Jules Verne, Amiens, France,
[2] Department of Biology, Faculty of Sciences, Ibn Zohr University, Agadir, Morocco

OPEN ACCESS

Edited by:
Sébastien Roger,
Université de Tours, France

Reviewed by:
David Crottès,
University of California, San Francisco, United States
Nelson Shu-Sang Yee,
Penn State Milton S. Hershey Medical Center, United States

***Correspondence:**
Halima Ouadid-Ahidouch
halima.ahidouch-ouadid@u-picardie.fr

Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *02 June 2020*
Accepted: *16 September 2020*
Published: *16 October 2020*

Citation:
Schnipper J, Dhennin-Duthille I, Ahidouch A and Ouadid-Ahidouch H (2020) Ion Channel Signature in Healthy Pancreas and Pancreatic Ductal Adenocarcinoma. Front. Pharmacol. 11:568993. doi: 10.3389/fphar.2020.568993

Pancreatic ductal adenocarcinoma (PDAC) is the fourth most common cause of cancer-related deaths in United States and Europe. It is predicted that PDAC will become the second leading cause of cancer-related deaths during the next decades. The development of PDAC is not well understood, however, studies have shown that dysregulated exocrine pancreatic fluid secretion can contribute to pathologies of exocrine pancreas, including PDAC. The major roles of healthy exocrine pancreatic tissue are secretion of enzymes and bicarbonate rich fluid, where ion channels participate to fine-tune these biological processes. It is well known that ion channels located in the plasma membrane regulate multiple cellular functions and are involved in the communication between extracellular events and intracellular signaling pathways and can function as signal transducers themselves. Hereby, they contribute to maintain resting membrane potential, electrical signaling in excitable cells, and ion homeostasis. Despite their contribution to basic cellular processes, ion channels are also involved in the malignant transformation from a normal to a malignant phenotype. Aberrant expression and activity of ion channels have an impact on essentially all hallmarks of cancer defined as; uncontrolled proliferation, evasion of apoptosis, sustained angiogenesis and promotion of invasion and migration. Research indicates that certain ion channels are involved in the aberrant tumor growth and metastatic processes of PDAC. The purpose of this review is to summarize the important expression, localization, and function of ion channels in normal exocrine pancreatic tissue and how they are involved in PDAC progression and development. As ion channels are suggested to be potential targets of treatment they are furthermore suggested to be biomarkers of different cancers. Therefore, we describe the importance of ion channels in PDAC as markers of diagnosis and clinical factors.

Keywords: ion channels, exocrine pancreas, pancreatic ductal adenocarcinoma, signaling pathways, biomarkers

INTRODUCTION

Ion channels are plasma membrane spanning proteins found in all human tissues, allowing rapid transport of ions and fluids between the extracellular and intracellular milieu (Niemeyer et al., 2001; Gouaux and Mackinnon, 2005). Opening of ion channels can result in redistribution of different ions, which changes the electrical and chemical properties of the cell leading to several cellular processes (Roux, 2017). These include multiple signal transduction and downstream signaling events, including regulation of gene expression, secretion of enzymes and hormones, and intracellular communication between compartments (Chen et al., 1994; Tolon et al., 1996; Stock and Schwab, 2015). A stable regulation of these processes maintains normal tissue homeostasis, such as cell cycle progression, migration, and apoptosis (Kunzelmann, 2005; Kunzelmann, 2016; Prevarskaya et al., 2018; Anderson et al., 2019). Accordingly, dysregulated expression as well as altered function of ion channels are related to a great number of diseases (Kim, 2014), and can drive the transformation from normal to malignant cell behavior (Litan and Langhans, 2015). Over the past decades, aberrant and even cancer-specific expression of numerous ion channels have been demonstrated in various types of cancers (Pedersen and Stock, 2013; Djamgoz et al., 2014). Together, the abnormal expression and activity of ion channels can be categorized as "hallmarks of cancer" (Hanahan and Weinberg, 2011).

The pancreas is a complex organ, which has two main functions exerted by an exocrine and endocrine compartment (Pandiri, 2014). Dysregulation of exocrine pancreatic fluid secretion can contribute to pathologies such as pancreatitis and neoplasms such as pancreatic ductal adenocarcinoma (PDAC), whereas a well-known disorder related to dysfunction of the endocrine pancreas is diabetes mellitus (Pallagi et al., 2015; Kirkegard et al., 2017). The exocrine pancreas ensures enzymatic secretion for digesting fats and proteins in the intestines and, in parallel, the secretion of abundant fluid rich in bicarbonate ions, which serves to neutralize the acidic chime in the duodenum (Ishiguro et al., 2012; Lee et al., 2012; Pallagi et al., 2015). The bicarbonate secretion involves a tightly coordinated network of ion channels and transporters (Novak et al., 2013). The ductal epithelial cells comprising the exocrine pancreas are, as other types of epithelia, well-organized and exhibit epithelial features such as a polarized morphology and specialized cell-to-cell contact with tight junctions (Rodriguez-Boulan and Nelson, 1989). The ductal cells are equipped with a highly polarized set of ion channels and transporters, enabling the net bicarbonate excretion at the apical membrane, balanced by the net efflux of acid *via* the basolateral membrane to maintain their intracellular pH (Steward et al., 2005). Therefore, a correct distribution of ion channels and transporters is important to maintain the secreting function of exocrine pancreas (Lee et al., 2012). Moreover, expression, function, and localization of ion channels in the plasma membrane are involved in the development and progression of PDAC (Pedersen et al., 2017). PDAC can arise from ductal cells (Schneider et al., 2005) or from acinar cells transforming to ductal cells by acinar–to-ductal-metaplasia, resulting in these cells possessing a ductal phenotype (Aichler et al., 2012). The transformation-associated loss of cell polarity and cell-cell adhesions of the epithelial cell layer will result in an altered localization of ion channels (Coradini et al., 2011; Pedersen and Stock, 2013).

Several reports and reviews about the role of transporters in bicarbonate, pancreatic fluid secretion and PDAC have been published (Novak, 2000; Lee et al., 2001; Novak et al., 2011; Ishiguro et al., 2012; Lee et al., 2012; Kong et al., 2014; Lemstrova et al., 2014; Pedersen et al., 2017; Yamaguchi et al., 2017). However, the role of ion channels in exocrine pancreas and in PDAC is not well understood. In this review, we aim to make a synthesis of the important role of ion channels and their localization and function in fluid secretion in healthy exocrine pancreatic tissue (see **Table 1** and **Figure 1**). Next, we summarize the sparse knowledge of the involvement of ion channels in PDAC progression and development *via* effects on proliferation, apoptosis, invasion and migration (see **Table 2** and **Figure 2**). Finally, we describe how ion channels are important novel biomarkers in PDAC (see **Table 2** and **Figure 3**).

EXPRESSION, LOCALIZATION, AND ROLE OF ION CHANNELS IN HEALTHY PANCREATIC EPITHELIAL CELLS

Potassium Channels

The relevance of K^+ channels in the exocrine pancreas received great attention in the 1970s–1980s, notably by Petersen's team, thanks to electrophysiological studies of ion channels on acinar pancreatic epithelial cells dissociated from the pancreas of different animal species. While several studies have shown the expression of different families of K^+ channels on both acinar and duct pancreatic cells (Petersen and Findlay, 1987; Bleich and Warth, 2000; Thevenod, 2002; Hayashi and Novak, 2013; Venglovecz et al., 2015), few studies have shown their physiological role in exocrine secretion.

Two excellent reviews have summarized the role of these channels in physiological process of ductal fluid secretion, likely by contributing to maintain the membrane potential and thereby providing driving forces for anion transport (Hayashi and Novak, 2013; Venglovecz et al., 2015). Among these channels the voltage- and Ca^{2+}-activated K^+, big conductance (BK, maxi-K), and the intermediate (IK, KCa3.1) Ca^{2+}-activated K^+ channels have been intensely studied in pancreatic ductal cells. The BK which is activated by cAMP and PKA is found on the basolateral membrane of rat pancreatic duct cells (Gray et al., 1990a). The authors suggest its role in pancreatic bicarbonate secretion. BK is also found mainly expressed in the apical membrane of guinea-pig non-transformed pancreatic duct epithelial cells (PDEC) (Venglovecz et al., 2011) where it regulates the bicarbonate secretion stimulated by the bile acid chenodeoxycholate likely through changes of the membrane potential. KCa3.1 was first characterized in cultured PDEC where it is expressed on the basolateral membrane of duct

TABLE 1 | Expression, localization, and the potential role of ion channels in exocrine pancreas.

Channel type	Species	Pancreatic Acini	Pancreatic Duct	Localization	Function	Ref.
K^+ Channels						
Kir2, Kir2.3, Kir7.1, Kir1.3	Rat	+		Basolateral	Unknown	(Kim et al., 2000) (Shuck et al., 1997)
Kir5.1 & Kir4.2	Rat Human	+	+	Unknown	Kir5.1 forms heteromeric channels with Kir4.2. Might have a role in the pH-dependent regulation of K^+ fuxes?	(Pessia et al., 2001) (Liu et al., 2000)
TALK-1 & TALK-2	Human	+		Unknown	Highly modulated (activation) by NOS and ROS	(Duprat et al., 2005) (Girard et al., 2001)
TASK-2	Human	+	+	Luminal in duct	Might drive the force for electrogenic HCO3– secretion?	(Duprat et al., 1997) (Duprat et al., 2005) (Hayashi and Novak, 2013)
minK	Rat	+		Unknown in acinar		(Kim and Greger, 1999)
KCNQ1(KvLQT1/ Kv7.) & KCNE1 (minK)	Mouse Rat	+	+	Luminal in duct Lateral and basolateral in acini	Cell volume regulation in ducts Membrane potential in acini Electrolyte/enzyme secretion with minK	(Kottgen et al., 1999) (Warth et al., 2002) (Warth and Barhanin, 2002) (Demolombe et al., 2001) (Hayashi et al., 2012)
KCa1.1 (BK, maxi-K)		+	+	Basolateral in acini/Luminal in duct	Activate luminal secretion	(Gray et al., 1990a) (Hede et al., 1999) (Hede et al., 2005)
BK (maxi-K)	Pig		+	Basolateral	Cl^- secretion	(Iwatsuki and Petersen, 1985a) (Iwatsuki and Petersen, 1985b) (Suzuki et al., 1985) (Petersen and Findlay, 1987) (Gallacher et al., 1984)
BK (maxi-K)	Human		+	Unknown		(Petersen et al., 1985)
BK (maxi-K)	Guinea-pig	+		Luminal	Regulation of HCO_3^- secretion-induced by the bile acid Chenodeoxycholate	(Venglovecz et al., 2011)
BK (maxi-K)	Rat	+		Basolateral	Might regulate membrane potential hyperpolarzation?	(Gray et al., 1990a) (Hede et al., 2005)
IK1 (KCa3.1)	Dog (cell lines, PDEC)	+	+	Basolateral/Luminal in duct	Driving force for Cl^- efflux Regulate HCO_3^- secretion Hyperpolarization	(Nguyen and Moody, 1998) (Thompson-Vest et al., 2006) (Jung et al., 2006)
IK1 (KCa3.1)	Mouse & Human		+	Luminal/basolateral	Setting the RMP. Involvement in anion and K^+ transport in stimulated ducts	(Hayashi et al., 2012)
IK1 (KCa3.1)	Human & Rat					(Ishii et al., 1997) (Joiner et al., 1997) (Hede et al., 2005)
Kv3.4	Mouse	+		Unknown	Unknown	(Kalman et al., 1998)
Kv1.5	Human	+		Unknown	Might regulate membrane potential?	(Bielanska et al., 2009) (Venglovecz et al., 2015)
Kv11.1 (ERG1)	Human		+	Luminal	Might regulate membrane potential?	(Hayashi et al., 2012)
Kv10.2	Human		+	Luminal	Might regulate membrane potential?	(Hayashi et al., 2012)
Calcium						
ORAI1	Mouse	+		Both in acinar Mostly basolateral	Might drive exocytosis of secretory granules or stimulate fluid and electrolyte secretion?	(Lur et al., 2009) (Hong et al., 2011)
ORAI2	Dog		+	Unknown	Unknown	(Kim et al., 2013)
ORAI3	Dog		+	Basolateral	Unknown	(Kim et al., 2013)
STIM1	Mouse	+		Both in acinar Mostly basolateral	Might drive exocytosis of secretory granules or stimulate fluid and electrolyte secretion?	(Lur et al., 2009) (Hong et al., 2011)
TRPC1	Mouse	+	+	Lateral side of the basolateral membrane	Might drive exocytosis of secretory granules or stimulate fluid and electrolyte secretion?	(Hong et al., 2011)
TRPC3	Mouse	+	+	Basolateral in acini	Might drive exocytosis of secretory granules or stimulate fluid and electrolyte secretion?	(Kim et al., 2006) (Kim et al., 2009)

(Continued)

TABLE 1 | Continued

Channel type	Species	Pancreatic Acini	Pancreatic Duct	Localization	Function	Ref.
TRPC6	Mouse	+		Unknown	Might drive exocytosis of secretory granules or stimulate fluid and electrolyte secretion?	(Kim et al., 2006)
TRPV6	Dog		+	Unknown	Unknown	(Kim et al., 2013)
TRPM7	Zebra fish		+	Luminal	Regulating epithelial cell-cycle progression, growth, and, consequently, acinar and ductal morphogenesis, also during embryogenesis	(Yee et al., 2011)
TRPM8	Human	+	(To some extend)	Unknown	Unknown	(Yee et al., 2010)
Chloride						
CFTR	Rat Guinea-pig Dog Human	+	+	Luminal in ducts	Drive fluid and HCO_3^- secretion	(Gray et al., 1988) (Gray et al., 1989) (Gray et al., 1990b) (Marino et al., 1991; Gray et al., 1993) (Zeng et al., 1997)
CaCC (TMEM16A)	Mouse Rat Guniea-pig Dog Bovine	+	+	Luminal on both	Drive fluid and HCO_3^- secretion	(Park et al., 2001) (al-Nakkash and Cotton, 1997) (Winpenny et al., 1995) (Nguyen et al., 1997) (Yokoyama et al., 2019) (Zdebik et al., 1997) (Wang and Novak, 2013) (Park et al., 2001) (Yang et al., 2008) (Bergmann et al., 2011) (Ousingsawat et al., 2009) (Huang et al., 2009) (Randriamampita et al., 1988) (Kasai and Augustine, 1990) (Marty et al., 1984) (Gray et al., 1989) (Gray et al., 1990b)
Sodium						
ENaC (δ -subunit)	Mouse Human	+	+	Luminal	Unknown	(McDonald et al., 1994; Waldmann et al., 1995; Zeiher et al., 1995; Novak and Hansen, 2002; Pascua et al., 2009) (Waldmann et al., 1995)
Other						
AQP1	Mouse Rat Human	+	+	Both on ducts	Facilitates water flow	(Gabbi et al., 2008) (Ko et al., 2002) (Furuya et al., 2002) (Burghardt et al., 2003)
AQP5	Mouse Rat Human		+	Luminal	Facilitates water flow	(Hurley et al., 2001) (Ko et al., 2002) (Burghardt et al., 2003)
AQP8	Rat Human	+		Luminal	Facilitates water flow	(Hurley et al., 2001) (Burghardt et al., 2003) (Koyama et al., 1997)
AQP12	Mouse Chicken Human	+		Intracellular	Synthesis of digestive enzymes	(Itoh et al., 2005) (Isokpehi et al., 2009)

epithelial cells (Nguyen and Moody, 1998). Activation of P2Y2R induced an increase of free intracellular calcium ($[Ca^{2+}]_i$) that activates KCa3.1, which in turn hyperpolarized the membrane potential, leading to a Cl^-dependent bicarbonate secretion (Jung et al., 2006). KCa3.1 was also found located on the basolateral and luminal membrane of pancreatic mouse and human duct cells (Hayashi et al., 2012). The same authors demonstrated that both luminal and basolateral KCa3.1 channels were involved in the regulation of membrane potential.

In acinar cells, the membrane potential created by K^+ channels, and waves of $[Ca^{2+}]_i$ provide the necessary driving force for Cl^- efflux through the luminal membrane, which is a key

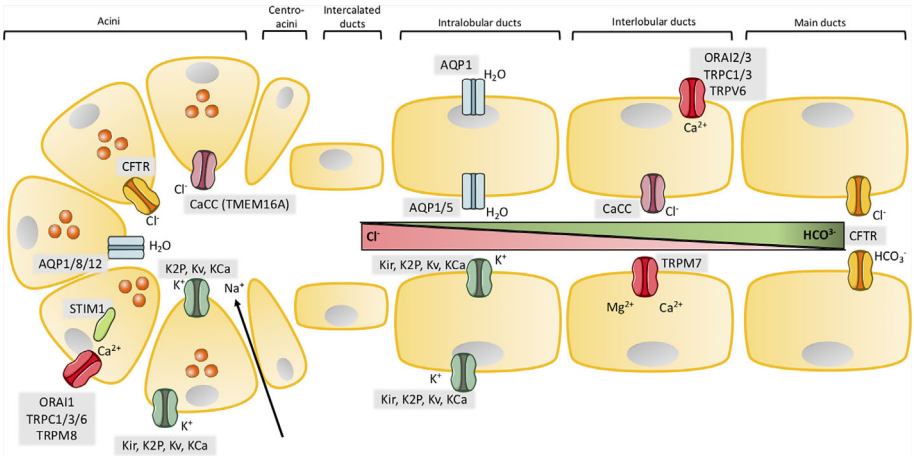


FIGURE 1 | Ion channels in exocrine pancreas. Illustration of the structure of acinar and major ductal segments of secretory glands in pancreas. Acinar cells secrete digestive enzymes (orange circles in acini) and an isotonic NaCl rich fluid which transports the enzymes to the ducts. Fluid secretion in acini cells is regulated by a Cl^- secretion process. Cl^- secretion is activated by $[Ca^{2+}]_i$, from a Ca^{2+} influx through SOCs in the basolateral membrane, where Cl^- channels, Ca^{2+} activated Cl^- channels (CaCC) and different types of K^+ channels are activated to provide the efflux of their respected ions. K^+ channels also create a driving force by maintaining a negative membrane potential. The negative charge mediated by a high concentration of Cl^- ions results in transport of Na^+ through tight junctions to the luminal space. NaCl makes the driving force for water to efflux through aquaporins and a cell shrinkage. This cell shrinkage reduces $[Ca^{2+}]_i$, which inhibits Cl^- and K^+ efflux through their channels and in parallel activates basolateral transporters and pumps to restore both Cl^- and K^+. The digestive enzymes are transported in the NaCl isotonic fluid to the ducts, which is low in HCO_3^- concentration in the proximal ducts, but this concentration increases through the transport to the distal duct cells. The ductal fluid becomes rich in HCO_3^-, by a two-step process. The first step takes place in the proximal ducts, where Cl^-/HCO_3^- exchangers secretes HCO_3^- and absorb Cl^- and Cl^- channels recycle Cl^-. As in the acinar cells an osmotic reaction happens, where efflux of negative HCO_3^- and Na^+ drives water flow through aquaporins. This results in high concentration of HCO_3^- (~100 mM), a low concentration of Cl^- (~25 mM) and a high fraction of water in the pancreatic juice. The second step takes place in the distal part of the ducts, where the specific Cl^- channel CFTR changes selectivity to HCO_3^- and function as a HCO_3^- efflux channel to determine the final concentration of the HCO_3^- rich fluid (~140 mM). K^+ channels may, as in acini, take part in the secretion of K^+ and regulation of anion transport by maintaining the membrane potential in both the basolateral and luminal membrane. SOCs ensure the influx of Ca^{2+} which takes part in regulation of ion channels through $[Ca^{2+}]_i$ as in acini. Activation or inhibition of P2 receptors by Ca^{2+} signaling also regulate anion secretion through K^+ and Cl^- channels.

TABLE 2 | Profile expression of ion channels in pancreatic ductal adenocarcinoma (PDAC) cell lines and tissue and how they are involved in driving PDAC formation and how channel expression correlate with clinical factors.

Channel	Profile expression Up/ Downregulated (method used for expression profiling)	Cell line/Solid tumor	Driving PDAC formation in form of	Downstream regulation and signaling	Channel expression correlates with clinical factors	Ref.
Potassium						
KCa3.1	Upregulated (mRNA, IHC, Microarray, electrophysiological, transcriptome data, TCGA analysis)	BxPC-3 Capan-1 MiaPaCa-2 PANC-1 Solid tumors	Proliferation Cell cycle progression Migration Invasion	Ras Oxidative Phosphorylation	High expression: Low overall survival Advanced tumor stage	(Bonito et al., 2016) (Jager et al., 2004) (Hayashi et al., 2012) (Jiang et al., 2017) (Shen et al., 2017) (Kovalenko et al., 2016) (Zaccagnino et al., 2016)
KCa4.1	mRNA	Capan-1 PANC-1				(Hayashi et al., 2012)
KCa4.2	mRNA	CFPAC PANC-1				(Hayashi et al., 2012)
$K_v1.3$	Downregulated in tumors (mRNA, IHC) Upregulated in cell lines (mRNA, protein)	AsPC-1 BxPC-3 Capan-1 Colo357 MiaPaCa-2 Panc-89	Apoptosis Hypermethylation		Low expression: Hypermethylation correlates with survival (not significant)	(Brevet et al., 2009) (Zaccagnino et al., 2017) (Bielanska et al., 2009)

(Continued)

TABLE 2 | Continued

Channel	Profile expression Up/ Downregulated (method used for expression profiling)	Cell line/Solid tumor	Driving PDAC formation in form of	Downstream regulation and signaling	Channel expression correlates with clinical factors	Ref.
		Panc-TUI Solid tumors				
$K_v1.5$	Upregulated (IHC)	Solid tumors				(Bielanska et al., 2009)
$K_v7.1$	Downregulated (Microarray, Nanostring electrophysiological)	A818–6 HPAF Solid tumors				(Tawfik et al., 2020) (Fong et al., 2003) (Zaccagnino et al., 2016)
minK	Downregulated (Microarray)	Solid tumors				(Zaccagnino et al., 2016)
$K_v10.1$	Unknown	Solid tumors	Tumor growth	Blocking shows antitumor activity		(Gomez-Varela et al., 2007) (Pardo and Stuhmer, 2014)
$K_v11.1$	Upregulated (mRNA, protein, IHC, Sequencing analysis)	BxPC-3 CFPAC-1 MiaPaCa-2 PANC-1 SW1990 T3M4 Solid tumors	Proliferation Cell cycle progression Migration Invasion Metastasis	miR96 EGFR-pathway ERK1/2 F-actin assembly	High expression: Low overall survival High Ki67 expression Advanced tumor grade	(Manoli et al., 2019) (Lastraioli et al., 2015b) (Zhou et al., 2012) (Feng et al., 2014) (Sette et al., 2013)
TREK-1	Upregulated (protein, electrophysiological)	AsPC-1 BxPC-3 Capan-1	Proliferation Migration	pH/Vm activated		(Sauter et al., 2016)
TASK-1	Downregulated (Microarray database analysis)	Solid tumors				(Williams et al., 2013)
TASK-2	(mRNA, protein electrophysiological)	HPAF				(Fong et al., 2003)
TWIK-1	Upregulated (Microarray database analysis)	Solid tumors				(Williams et al., 2013)
TWIK-3	Downregulated (Microarray)	Solid tumors	Cell differentiation			(Zaccagnino et al., 2016)
Kir3.1	Upregulated (mRNA, IHC)	Solid tumors				(Brevet et al., 2009)
Kir4.2	Downregulated (Microarray)	Solid tumors	Cell differentiation			(Zaccagnino et al., 2016)
Kir5.1	Downregulated (Microarray)	Solid tumors				(Zaccagnino et al., 2016)
Sodium						
VGSC SCN9A SCNA3	Downregulated (Microarray)	MiaPaCa-2 CAV Solid tumors	Proliferation	Inhibition of growth with phenytoin		(Sato et al., 1994; Koltai, 2015) (Zaccagnino et al., 2016)
ASIC1	Upregulated (mRNA, protein, IHC)	AsPC-1 BxPC-3 PANC-1 SW1990 Solid tumors	EMT Metastasis	RhoA		(Zhu et al., 2017)
ASIC3	Upregulated (mRNA, protein, IHC)	AsPC-1 BxPC-3 PANC-1 SW1990 Solid tumors	EMT Metastasis	RhoA		(Zhu et al., 2017)
Calcium						
ORAI1	Different expression in different cell lines (mRNA, protein) Downregulated (Microarray)	AsPC-1 BxPC-3 Capan-1 MiaPaCa-2 PANC-1 Solid tumors	Apoptosis Proliferation	Calcium-regulated Akt/ mTOR/NFAT signaling		(Kondratska et al., 2014) (Khan et al., 2020) (Zaccagnino et al., 2016)

(Continued)

TABLE 2 | Continued

Channel	Profile expression Up/ Downregulated (method used for expression profiling)	Cell line/Solid tumor	Driving PDAC formation in form of	Downstream regulation and signaling	Channel expression correlates with clinical factors	Ref.
STIM1	Different expression in different cell lines Upregulated in chemo-resistant cells (mRNA, protein,TCGA analysis, IHC)	AsPC-1 BxPC-3 Capan-1 CFAPC-1 MiaPaCa2 Panc-1 Solid tumors	Apoptosis Proliferation Invasion EMT Gemcitabine resistance	Regulated by HIF1-alpha	High expression: Low disease-free survival Advanced tumor grade	(Kondratska et al., 2014) (Zhou et al., 2020) (Wang et al., 2019)
TRPM2	Upregulated (mRNA, TCGA analysis)	PANC-1 Solid tumors	Proliferation Migration Invasion		High expression: Low overall survival	(Lin et al., 2018) Reviewed in: (Stoklosa et al., 2020)
TRPM7	Upregulated (mRNA, protein, IHC, electrophysiological)	BxPC-3 Capan-1 HPAF-II MiaPaCa2 PL45 Panc-1 Panc 02.03 Solid tumors	Proliferation Cell cycle progression Migration Invasion	Mg^{2+}-sensitive Socs3a-pathway Hsp90α/uPA/ MMP-2 proteolytic axis	High expression: Low overall survival Advanced tumor grade Advanced tumor stage Large tumor size Metastasis Molecular phenotype Treatment response	(Yee et al., 2011; Yee et al., 2012a; Yee et al., 2015) (Rybarczyk et al., 2012; Rybarczyk et al., 2017)
TRPM8	Upregulated (mRNA, protein, IHC, electrophysiological)	BxPC-3 Capan-1 HPAF-II MiaPaCa2 Panc-1 Panc 02.03 PL45 Solid tumors	Proliferation Cell cycle progression Apoptosis Invasion Migration	Glycosylation states	High expression: Low overall survival Low disease-free survival Poor prognosis Metastasis Molecular phenotype Treatment response	(Yee et al., 2010) (Yee et al., 2012a) (Liu et al., 2018) (Yee et al., 2014) (Du et al., 2018) (Cucu et al., 2014) (Ulareanu et al., 2017)
TRPC1	(mRNA, protein)	BxPC-1 CAPAN-1 CFPAC PANC-1	Motility	TGF-β-induced Ca/PKCα signaling		(Kim et al., 2013) (Dong et al., 2010)
TRPC4	(mRNA, protein)	BxPC-1				(Dong et al., 2010)
TRPC6	(mRNA, protein)	BxPC-1				(Dong et al., 2010)
TRPV1	Upregulated (mRNA, protein, IHC)	Capan-1 MiaPaCa-2 PANC-1 Solid tumors	Proliferation Apoptosis	EGFR/MAPK		(Huang et al., 2020) (Hartel et al., 2006) Reviewed in: (Liddle, 2007)
TRPV6	Upregulated (mRNA, protein, IHC) Downregulated (Microarray, Nanostring)	A818-6 AsPC-1 BxPC-3 CAPAN-1 CFPAC PANC-1 SW1990 Solid tumors	Cell cycle Apoptosis Metastasis	Numb protein	High expression: Low overall survival Advanced tumor stage Large tumor size Vascular infiltration	(Kim et al., 2013; Song et al., 2018) (Tawfik et al., 2020) (Zaccagnino et al., 2016)
IP3R	Immunoblotting (IF)	PANC-1	Migration	Colocalization with STIM1/ ER–PM junctions		(Okeke et al., 2016)
CACNA1	Upregulated (Microarray)	Solid tumors				(Zaccagnino et al., 2016)
CACNA1G	Downregulated	Solid tumors				(Zaccagnino et al., 2016)
Chloride						
CLCA1	Upregulated (IHC, Proteomics)	Solid tumors	Unclear		Low expression correlates with poor prognosis Low overall survival	(Hu et al., 2018a) (Hu et al., 2018b; Hu et al., 2019)
CLCNKB	Downregulated (Microarray)	Solid tumors				(Zaccagnino et al., 2016)
CLCN1	Downregulated (Microarray)	Solid tumors				(Zaccagnino et al., 2016)

(Continued)

TABLE 2 | Continued

Channel	Profile expression Up/ Downregulated (method used for expression profiling)	Cell line/Solid tumor	Driving PDAC formation in form of	Downstream regulation and signaling	Channel expression correlates with clinical factors	Ref.
CLIC1	Upregulated (IHC)	MiaPaCa-2 PANC-1 Solid tumors	Proliferation Invasion		High expression: Low overall survival Advanced tumor grade Advanced tumor stage Large tumor size	(Jia et al., 2016) (Lu et al., 2015)
CLIC2	mRNA, protein, electrophysiological	HPAF				(Fong et al., 2003)
CLIC3	Upregulated (mRNA, protein, electrophysiological, immunohistochemistry)	HPAF Solid tumors	Promote integrin recycling		High expression: Low overall survival	(Dozynkiewicz et al., 2012) (Fong et al., 2003)
CLIC4	Upregulated (IHC)	Solid tumors	Invasion			(Zou et al., 2016)
CLIC5	(mRNA, protein electrophysiological) Downregulated (Microarray)	HPAF Solid tumors	Cell differentiation			(Fong et al., 2003) (Zaccagnino et al., 2016)
TMEM16A	Upregulated (mRNA, electrophysiological, TCGA analysis)	AsPC-1 BxPC-3 Capan-1 MiaPaCa-2 PANC-1 Solid tumors	Migration	TMEM16A-dependent store-operated calcium entry (SOCE). EGFR-signaling pathways	High expression: Low overall survival	(Sauter et al., 2015) (Wang and Novak, 2013) (Crottes et al., 2019)
TMEM16E	Upregulated (mRNA, protein, IHC)	BxPC-3 HPAC PANC-1 Solid tumors	Proliferation Migration			(Song et al., 2019)
TMEM16J	Upregulated (mRNA, protein, IHC)	AsPC-1 BxPC-3 Capan-2 PANC-1 Solid tumors	Proliferation	ERK1/2 EGFR	High expression: Low overall survival	(Jun et al., 2017)
CFTR	Downregulated (mRNA, Microarray, Sequencing analysis) Immunoblotting (IF)	AsPC-1 BxPC-3 Capan-1 Capan-2 Colo357 CFPAC1 HPAC HPAF HS766T MiaPaCa-2 PANC-1 QGP1 S2CP9 Suit2 SW1990 T3M4 Solid tumors Organoids	EMT	Regulate expression of MUC4	*CFTR* mutation leads to a higher risk of getting pancreatic cancer	(Chambers and Harris, 1993) (Singh et al., 2007) (McWilliams et al., 2010; Cazacu et al., 2018) (Hennig et al., 2019) (Zaccagnino et al., 2016)
Aquaporins						
AQP1	Upregulated (mRNA, protein, IHC)	Solid tumors			High expression: Low overall survival Advanced tumor stage Large tumor size Lymph node metastasis Tumor differentiation Invasion	(Burghardt et al., 2003) (Zou et al., 2019)
AQP3	Upregulated (mRNA, Microarray, protein, IHC)	BxPC-3 Capan-2 HPAC	Proliferation Apoptosis EMT	mTOR/S6 signaling Simultaneous overexpression of	High expression: Low overall survival Advanced tumor stage	(Burghardt et al., 2003) (Huang et al., 2017)

(Continued)

TABLE 2 | Continued

Channel	Profile expression Up/ Downregulated (method used for expression profiling)	Cell line/Solid tumor	Driving PDAC formation in form of	Downstream regulation and signaling	Channel expression correlates with clinical factors	Ref.
		HPAFII Solid tumors		EGFR, Ki-67, and CK7, down-regulation of E-cadherin and vimentin	Large tumor size Lymph node metastasis Tumor differentiation Invasion	(Zou et al., 2019) (Direito et al., 2017) (Zaccagnino et al., 2016)
AQP4	(mRNA)	Capan-1 Capan-2 Solid tumors				(Burghardt et al., 2003)
AQP5	Upregulated (mRNA, IHC)	Capan-1 Capan-2 HPAF Solid tumors	Proliferation Differentiation EMT	Simultaneous overexpression of EGFR, Ki-67, and CK7. Downregulation of E-cadhering and vimentin.	High expression: Low overall survival Tumor differentiation	(Burghardt et al., 2003) (Direito et al., 2017)
AQP8	mRNA Downregulated (Microarray)	Solid tumors				(Burghardt et al., 2003) (Zaccagnino et al., 2016)
Ionotropic receptors						
P2X4	mRNA	AsPC-1 BxPC-3 Capan-1 CFPAC-1 MiaPaCa-2 PANC-1				(Kunzli et al., 2007) (Hansen et al., 2008) (Giannuzzo et al., 2015)
P2X6	mRNA	AsPC-1 BxPC-3 Capan-1 CFPAC-1 MiaPaCa-2 PANC-1				(Kunzli et al., 2007) (Hansen et al., 2008) (Giannuzzo et al., 2015)
P2X7	Upregulated (mRNA, protein, IHC)	AsPC-1 BxPC-3 Capan-1 CFPAC-1 MiaPaCa-2 PANC-1 Solid tumors	Proliferation Apoptosis Invasion Migration	PKC, PLD ERK1/2, and JNK Decreased nitric oxide synthase		(Giannuzzo et al., 2015) (Kunzli et al., 2007) (Hansen et al., 2008) (Choi et al., 2018)
NMDAR	(IHC, Microarray)	BxPC-3 HPAFII SUIT2 Solid tumors	Proliferation Survival			(Li and Hanahan, 2013) (North et al., 2017)

step in initiating fluid and electrolyte secretion (Lee et al., 2012). The activation of K^+ channels located on the basolateral membrane hyperpolarizes the resting membrane potential, promoting the driving force for luminal Cl^- efflux through Cl^- channels (Petersen, 2005). A Ca^{2+}-dependent maxi- K^+ channel (200 pS) has been characterized upon stimulation with acetylcholine (ACh), cholecystokinin (CCK), and bombesin in pancreatic acinar cells (Maruyama et al., 1983; Iwatsuki and Petersen, 1985a; Iwatsuki and Petersen, 1985b; Petersen et al., 1985; Suzuki et al., 1985; Petersen and Findlay, 1987). Moreover, Pearson et al. (1984) showed, on isolated pancreas acinar pig cells, that neural and hormonal (ACh, bombesin and pentagastrin) stimulation evokes a Ca^{2+}-dependent cell hyperpolarization by causing an increase in membrane K^+ conductance (Pearson et al., 1984). An intermediate Ca^{2+}-activated K^+ channel is also expressed in both the basolateral and the apical membranes of acinar cells (Thompson-Vest et al., 2006), but its role has not been studied.

KCNQ1 (KVLQT1, Kv7.1) and KCNE1 (IsK, minK) have been found in abundance in pancreatic acinar cells (Kottgen et al., 1999; Bleich and Warth, 2000; Demolombe et al., 2001; Warth and Barhanin, 2002; Warth et al., 2002; Hayashi et al., 2012). By using mouse models associated with electrophysiological studies, Warth et al. (2002) showed that KCNQ1 was predominantly located at the basolateral membrane and its co-assemblage with KCNE1 leads to a voltage-dependent K^+ current that was increased by cholinergic stimulation and inhibited by the KCNQ1 blocker (Kim and Greger, 1999; Kottgen et al., 1999; Warth et al., 2002). The fact that inhibition of KCNQ1 channels diminishes intestinal Cl^- secretion, made the authors suggest its involvement in pancreatic electrolyte secretion process.

K^+ inwardly rectifying channels (Kir) channels are expressed in exocrine pancreas. Kir 2.1, Kir2.3, Kir7.1, Kir5.1, and Kir4.2 were detected in rat pancreatic acini (Kim et al., 2000; Pessia et al., 2001). *In-situ* hybridization analysis confirmed the expression of Kir5.1 in human pancreatic acinar and ductal

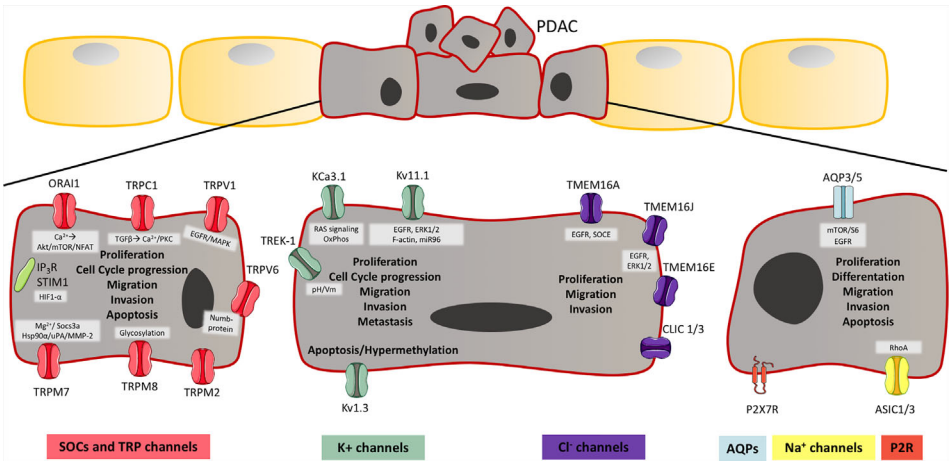


FIGURE 2 | Ion channels in pancreatic ductal adenocarcinoma (PDAC). Illustration of ion channels, which have been shown to have a role in hallmarks of cancer, thereby PDAC development and progression. As cancer cells lose their polarity, the localization of the channels is unknown, and on the illustration, it should be considered that the channels have no particular localization. The aberrant expression in PDAC cells, are shown for; Store-operated channels (SOCs) and transient receptor potential (TRP) channels, K^+ channels, Cl^- channels, aquaporins (AQP), Na^+ channels and P2X7R. These channels are known to be involved in PDAC development and progression through proliferation, cell cycle progression, differentiation, migration, invasion, metastasis, and apoptosis. The known pathways and mechanism, which have been shown to be involved in these processes are shown in a grey box next to the channel and are mentioned in **Table 2**. The channels shown to be expressed in PDAC, but where the role is unknown are also shown in **Table 2**.

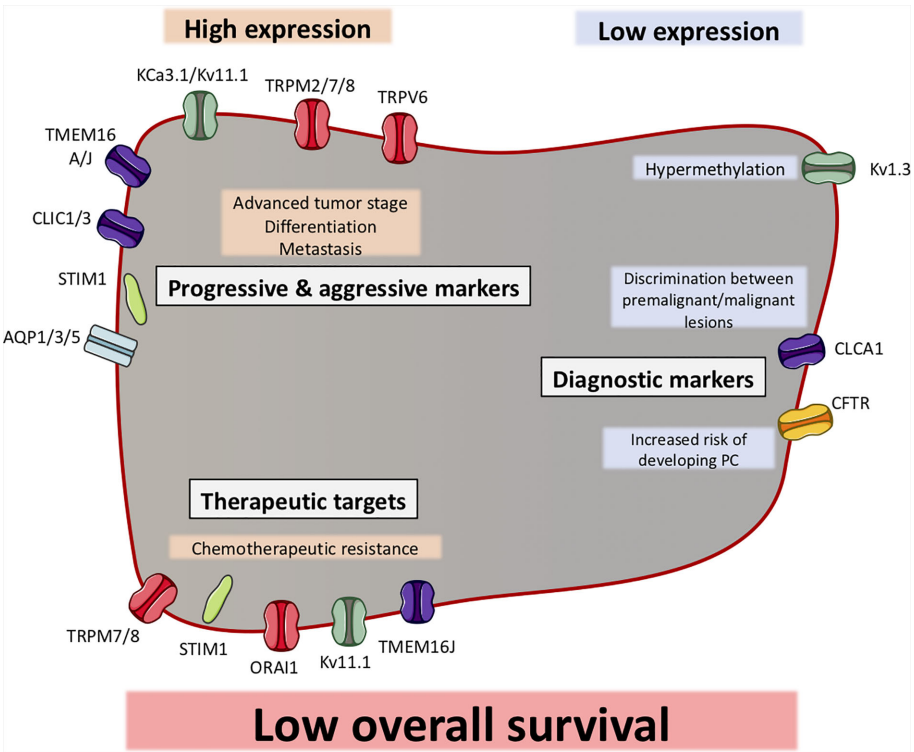


FIGURE 3 | Ion channels can function as biomarkers in pancreatic ductal adenocarcinoma (PDAC). Illustration of ion channels, where the expression has been shown to be correlated with clinical factors. Most of the ion channels show a high expression in PDAC, which correlates with clinical factors (indicated in grey boxes). Some ion channels have shown to be to have a low expression in PDAC, which correlates with other clinical factors. The ion channels are grouped as progression and aggressiveness markers, diagnostic markers or therapeutic targets. Among all ion channels, their expression (except CFTR) have been shown to be correlated with a low overall survival.

cells (Liu et al., 2000). Moreover, it has been suggested that Kir5.1 forms heteromeric channels with Kir4.2 in rat pancreas and is involved in the pH-dependent regulation of K^+ flux (Pessia et al., 2001). Kir1.3 was also detected by northern blot analysis, in human pancreas (Shuck et al., 1997). The 2-Pore K^+ channel (K_2P) family has also been found in human exocrine pancreas; however, their localization and function are still unknown. For example, TALK-1 and TALK-2 are very specifically expressed in exocrine pancreas where they are activated by NOS and ROS (Girard et al., 2001; Duprat et al., 2005), while TASK-2 is expressed in both exocrine and endocrine pancreas (Duprat et al., 1997; Duprat et al., 2005).

Calcium Channels

As Petersen and co-workers showed the relevance of K^+ channels in exocrine pancreas, they have also described the role of Ca^{2+} signaling, in pancreatic acinar cells (Petersen, 2014). In the early 70's they showed that movements of Ca^{2+} was evoked upon ACh stimulation released Ca^{2+} from intracellular stores and that only a small part of Ca^{2+} was taken up from the extracellular solution (Case and Clausen, 1973; Matthews et al., 1973). This Ca^{2+} signaling is involved in exocrine pancreatic fluid secretion as both acinar and duct cells in pancreas are regulated by receptors that change $[Ca^{2+}]_i$, which activates epithelial Ca^{2+}-dependent K^+ and Cl^- ion channels, thereby enzyme and fluid secretion (Petersen, 2014). The Ca^{2+} signal is initiated by ACh or CCK, binding to specific receptors (Case and Clausen, 1973; Matthews et al., 1973; Petersen and Ueda, 1976), which generates specific Ca^{2+} signals. These signals start by Ca^{2+} activating phospholipase C, which hydrolyzes PIP_2, hence generating IP_3 and diacylglycerol. IP_3 binds to IP_3 receptors located in the ER at the apical pole of the acinar cells mediating a Ca^{2+} wave to the basal pole (Mogami et al., 1997; Hong et al., 2011). This evokes a Ca^{2+} ER store depletion that results in clustering of the ER Ca^{2+} sensor STIM1, which activates store-operated channels (SOCs) and transient receptor potential (TRP) channels, leading to Ca^{2+} influx (Petersen and Tepikin, 2008). Members and regulators of SOCs are the SOC channel pore-forming ORAI proteins (ORAI1-3) and their regulators STIM (STIM1-2) (Hoth and Niemeyer, 2013). ORAI1 is the best described among these and are found to be expressed at the apical membrane of pancreatic acinar cells where it colocalizes with IP_3R (Hong et al., 2011; Lur et al., 2011) and at the basolateral membrane where it colocalizes with STIM1 (Lur et al., 2011). Recently, it has been shown that inhibition of ORAI1 in pancreatic acinar cells abolished SOC entry upon stimulation with thapsigargin, CCK, and the bile acid taurolithocholic acid 3-sulfate, indicating that ORAI1 mediates SOC entry in pancreatic acinar cells (Gerasimenko et al., 2013; Wen et al., 2015).

TRPC channels have also been found to participate or influence store-dependent Ca^{2+} influx in pancreatic acinar cells. TRPC1 was found to localize both at the apical and lateral regions of the basolateral membrane, and pancreatic acinar cells isolated from $TRPC1^{-/-}$ mice showed reduced Ca^{2+} influx and Ca^{2+} oscillation frequency (Hong et al., 2011). The role of TRPC1 in pancreatic acinar cells is not yet known, but it is suggested to have a similar role as in salivary glands, where they regulate fluid secretion and Ca^{2+} activated K^+ channels (Liu et al., 2007). TRPC3 was found in the junctional site of the apical pole and the basolateral membrane of pancreatic acini cells and in $TRPC3^{-/-}$ mice a reduction of Ca^{2+} influx was seen (Kim et al., 2006). Furthermore, TRPC6 seemed to be expressed in the pancreatic acini cells, but its localization and role are unknown (Kim et al., 2006). These data suggest that TRPC channels are involved in the SOC entry of pancreatic acini cells and could contribute to fluid secretion. Other TRP channels have been found to be expressed in exocrine pancreas; TRPV6 (Kim et al., 2013), TRPM7 (Yee et al., 2011) and TRPM8 (Yee et al., 2010). However, only the role of TRPM7 is described. In a zebra fish model, it has been found that TRPM7 is involved in the developmental processes of exocrine pancreas, which was linked to Mg^{2+} signaling (Yee et al., 2011). Diminish of cell cycle progression and cell growth in TRPM7-mutated zebra fish models attenuated proliferation of exocrine pancreatic epithelia. This was partially rescued by adding extra Mg^{2+} to the embryo medium (Yee et al., 2011). Furthermore, the proliferation was also regulated by suppressor of cytokine signaling 3a (socs3a), indicating that TRPM7 plays a role in the development of exocrine pancreas (Yee et al., 2011), but the physiological role in fluid secretion is yet to be determined.

In duct cells, HCO_3^- secretion is mediated by cAMP/Ca^{2+} signaling systems. Through specific Ca^{2+} channels and Ca^{2+} activated ion channels (Ca^{2+}-activated K^+ and Cl^- channels), Ca^{2+} can act as key player in regulation and secretion of pancreatic juices (Lee M. G. et al., 2012). The localization of SOCs in duct cells, due to HCO_3^- fluid secretion, is not well studied. However, it has been found that SOC-mediated Ca^{2+} influx can be a driving force for exocytosis, evoked by trypsin (Kim et al., 2008) in dog PDEC. The same authors have shown the function of SOCs in dog PDEC where the typical inward rectifying current was found, as for other types of epithelial cells (Kim et al., 2013). Furthermore, it was found that STIM1, STIM2, ORAI1, ORAI2, and ORAI3 as well as TRPC1 and TRPV6 are all expressed in dog PDEC, where ORAI3 was shown to be the dominant expressing type (Kim et al., 2013). Moreover, STIM1 and ORAI3 are colocalized in both single cell PDEC and polarized monolayers upon thapsigargin treatment (Kim et al., 2013). Using thapsigargin, the same authors showed an increased $[Ca^{2+}]_i$ only at the basolateral membrane, indicating that SOCs are mainly located at this site of the plasma membrane (Kim et al., 2013). It might be hypothesized that the localization of SOCs and Ca^{2+}-activated ion channels are the same in pancreatic duct cells as in acinar cells, and that they play a role in HCO_3^- secretion, as they play a role in enzyme and fluid secretion in acinar cells (Maleth and Hegyi, 2014).

Aquaporin Channels

Aquaporins (AQPs) are activated by Ca^{2+} and mediate a water flow through the luminal membrane. The role of some AQP types in physiological and pathophysiological processes of exocrine pancreas has already been reviewed (Burghardt et al., 2006; Delporte, 2014; Arsenijevic et al., 2019). AQP1 is expressed

at the apical and basolateral membrane of centro-acinar cells and intercalated ductal cells (Burghardt et al., 2003; Burghardt et al., 2006) and is also expressed in capillary endothelial cells and at the pancreatic zymogen granule membrane (Cho et al., 2002; Burghardt et al., 2003). AQP5 has been found to be co-localized with AQP1 in the apical membrane of centro-acinar cells and intercalated ductal cells (Burghardt et al., 2003). Otherwise, AQP8 is expressed only in acinar cells in the apical membrane (Isokpehi et al., 2009). In the two-step process of pancreatic fluid secretion, AQP8 in the pancreatic acinar cells ensures the water flow across the plasma membrane, where NaCl makes the driving force. In the pancreatic ductal cells the driving force is maintained by $HCO3^-$ and Na^+ through AQP1 and AQP5 (Burghardt et al., 2006). However, this theory is not well explained, since pancreatic fluid secretion was not found to be altered in AQP1, AQP5, AQP8, or AQP12 knockout mice (Ma et al., 2001; Yang et al., 2005; Ohta et al., 2009). Recently, the role of AQP1 has been confirmed to be involved in pancreatic fluid and bicarbonate secretion in an AQP1-knockout mouse model (Venglovecz et al., 2018).

Chloride Channels

In the early 80's the evidence for Ca^{2+} activated Cl^- channels (CaCC) were presented by whole-cell patch clamp and single-channel currents in rat lacrimal acinar. Marty and co-workers showed that Cl^- currents were evoked by muscarinic receptor activation and Ca^{2+}, as previously demonstrated for the K^+ current (Marty et al., 1984; Petersen, 1992). Shortly after, following investigations confirmed this Ca^{2+} activated Cl^- current in rat pancreatic acinar cells (Randriamampita et al., 1988). The localization of these Ca^{2+}-dependent channels was proposed to be both on the basolateral and the luminal site, but speculations and further studies revealed that the localization of CaCC was found in the luminal membrane of pancreatic acinar cells, where an early activation of Cl^- currents was seen upon ACh stimulation (Kasai and Augustine, 1990; Zdebik et al., 1997). A small delayed current was found after Ca^{2+} has spread to the basal pole of the cell, suggesting that CaCC are highly located at the luminal membrane and to some extent in the basolateral of pancreatic acinar cells (Kasai and Augustine, 1990). New evidence shows clearly that CaCC are exclusively localized to the apical membrane and regulate pancreatic fluid secretion (Marty et al., 1984; Kasai and Augustine, 1990; Park et al., 2001).

Gray and his team have investigated the properties and roles of Cl^- channels in pancreatic duct epithelial cells. They and others, found two types of Cl^- channels in pancreatic ducts cells; cystic fibrosis transmembrane conductance regulator (CFTR), regulated by rises in $[cAMP]_I$ and CaCC, regulated by an increase in $[Ca^{2+}]_i$ (Gray et al., 1989; Riordan et al., 1989; Gray et al., 1990b; Gray et al., 1993; al-Nakkash and Cotton, 1997; Nguyen et al., 1997). Both types of channels have been found in several species and to be localized in the apical membrane of duct cells (Gray et al., 1990b; Marino et al., 1991; Ashton et al., 1993; Evans et al., 1996; al-Nakkash and Cotton, 1997; Zeng et al., 1997; Ishiguro et al., 2002; Wang and Novak, 2013; Yokoyama et al., 2019). CaCC have been found in rodent pancreatic ducts. Here, it was shown that increases in $[Ca^{2+}]$i, evoked by either ionomycin or ACh activated the Cl^- channels (Gray et al., 1990b; Gray et al., 1995). Furthermore, Cl^- currents were detected in mouse pancreatic ducts with no detectable function of CFTR, which indicates that these currents are carried by an ion channel that is distinct from CFTR (Gray et al., 1994; Winpenny et al., 1995).

Until now, it has been shown that mammalian TMEM16 proteins have different physiological functions. TMEM16A and B are suggested to be CaCC, where both of TMEM16E and F are suggested to have scramblase and channel activities. TMEM16D, G, and J are suggested to only have a scramblase activity. Therefore, the channel nature of all TMEM16 proteins is still not clearly identified [Reviewed in (Falzone et al., 2018)]. Recently, it has been suggested that TMEM16A, of the TMEM16/Anoctamin family, is the CaCC gene candidate for Cl^- secretion (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008). In rodent pancreatic acinar cells and intercalated ducts, expression of TMEM16A was found by immunostaining and RT-PCR (Huang et al., 2009; Yokoyama et al., 2019). The biophysical properties of the channel agreed with Ca^{2+}-dependent Cl^- currents, described elsewhere (Yang et al., 2008). Another study demonstrated that Ca^{2+}-dependent Cl^- secretion was defective in acinar cells from TMEM16A-null mice, indicating that TMEM16A has a physiological role in pancreatic fluid secretion (Ousingsawat et al., 2009).

The model of how Cl^- is secreted through channels in exocrine pancreas is described as a two-step process, starting by the activation by ACh or CCK, which trigger an IP_3-mediated rise of cytosolic Ca^{2+} (Iwatsuki and Petersen, 1977; Reubi et al., 2003; Gautam et al., 2005; Wang and Cui, 2007). In response to this stimulation, the NaCl rich fluid starts to be produced (Hegyi and Petersen, 2013). At the basolateral membrane the Na^+-K^+-$2Cl^-$ cotransporters (NKCC), Cl^-/$HCO3^-$ exchangers and Na^+/K^+ pumps are activated, to function together to establish the Cl^- uptake mechanism. The increased $[Ca^{2+}]_i$ enhances the Cl^- conductance of the luminal membrane and a K^+ channel-mediated hyperpolarization of the basolateral membrane creates the driving force for Cl^- efflux to the luminal space. At the apical membrane, Cl^- ions pass through the Cl^- channels. This hormonal stimulation by ACh and CCK, leading to increased $[Ca^{2+}]_{i,}$ plays the central role in activating enzyme release and electrogenic Cl^- secretion (Petersen and Gallacher, 1988; Mogami et al., 1997; Giovannucci et al., 2002; Petersen, 2005). While Cl^- passes through the acinar cells a negative charge in the luminal space arises, which moves Na^+ from the interstitial space to the acinar lumen *via* the paracellular pathway through leaky tight junctions, resulting in NaCl secretion. In physiological circumstances the acinar luminal Cl^- concentration contains 135 mM Cl and 25 mM HCO3- (Park et al., 2010). The second step in pancreatic fluid secretion occurs in the duct cells and depends on the high concentration of luminal Cl^- as it activates HCO_3^- efflux through Cl^-/HCO_3^- exchangers, which elevates the luminal HCO_3^- concentration and thereby activates CFTR functioning to secrete

Cl^- and to some extend HCO_3^- (Ishiguro et al., 2009; Wilschanski and Novak, 2013). The HCO_3^- concentration in the fluid increases along the ducts, while the Cl^- concentration reciprocally decreases. By the time the pancreatic fluid leaves the ducts the ratio is inverse, with the HCO_3^- concentration around 140 mM and the Cl^- concentration around 20 mM (Park et al., 2010). These specific concentrations will inhibit CFTR and Cl^-/HCO_3^- exchangers to prevent HCO_3^- reabsorption (Wright et al., 2004).

Sodium Channels

The efflux of Na^+ through tight junctions in both the acinar and ductal cells is a part of regulating the HCO_3^- rich fluid to be isotonic and to keep the cell osmolarity (Lee M. G. et al., 2012). The expression and function of Na^+ channels in normal pancreatic tissue are controversial. Some studies have shown functional expression of amiloride sensitive epithelial sodium channels (ENaC) in interlobular ducts from mice (Zeiher et al., 1995; Pascua et al., 2009). Moreover, transcripts of different subunits of ENaC have been also detected in human pancreas (McDonald et al., 1994; Waldmann et al., 1995; Novak and Hansen, 2002). Other studies have shown no functional activity of ENaC in isolated small ducts from rats or in PDAC cell lines Capan-1 and HPAF (Novak and Hansen, 2002; Fong et al., 2003; Wang and Novak, 2013), which is in accordance with the secretory nature of pancreatic ducts.

EXPRESSION OF ION CHANNELS IN PDAC CELLS AND HUMAN TISSUES, FUNCTION, AND ASSOCIATED SIGNALING PATHWAYS IN CELL LINES

Potassium Channels in PDAC

Kv Channels

It is widely accepted that Kv channels participate in cancer development and progression and their expression has shown to be aberrant in several types of tumor tissue, also in PDAC (Serrano-Novillo et al., 2019; Teisseyre et al., 2019). It has been shown that Kv1.3 is expressed in different human PDAC cell lines, harboring mutation in p53 (Zaccagnino et al., 2017). The authors demonstrate that the inhibition of Kv1.3 by clofazimine, induces apoptosis *in-vitro* and reduces tumor weight *in-vivo* (Zaccagnino et al., 2017). Another study has reported a remodeling of Kv1.3 and Kv1.5 on a large cohort of human tissue samples (Bielanska et al., 2009). In fact, they showed that protein expression of Kv1.3 was lower in PDAC tissue, while Kv1.5 had a higher protein expression in PDAC tissue compared to healthy tissue (Bielanska et al., 2009; Comes et al., 2013). The low expression of Kv1.3 in PDAC can be explained by a hypermethylation of the *KCNA3* gene promoter (Brevet et al., 2009). Similar to Kv1.3 the expression of Kv7.1 has recently been shown to be down-regulated in PDAC (Zaccagnino et al., 2016; Tawfik et al., 2020). *KCNQ1* (gene coding for Kv7.1) was downregulated in PDAC tissue, compared to normal tissue. In addition, downregulation of *KCNQ1* was found in a system comparing PDAC A818–6 cells grown as a highly malignant undifferentiated monolayer (ML) or as three-dimensional (3D) single layer hollow spheres (HS). Database analysis showed that *KCNQ1* was involved in the enrichment of pancreatic secretion in normal pancreatic epithelium and HS, suggesting that a downregulation of *KCNQ1* might impair fluid secretion in PDAC and ML cells, while being maintained in normal pancreas and HS cells (Tawfik et al., 2020). Another comprehensive study has been investigating the gene-expression levels of the transportome in PDAC and normal specimens (Zaccagnino et al., 2016). The authors showed the downregulation of five different K^+ channels, including the K^+ voltage-gated channels; *KCNQ1* and *KCNE1*. Moreover, their results showed a downregulation of genes coding for the Kir4.2 (*KCNJ15*), Kir5.1 (*KCNJ16*), and the K_2P channel TWIK-3 (*KCNK7*). In addition, the expression of *KCNJ15* and *KCNK7* was associated with the expression of EMT transcription factors (Zaccagnino et al., 2016). The authors also suggested that the higher expression of K^+ channels in normal pancreatic epithelium takes part in setting the resting membrane potential, which generates the driving force of fluid and ion secretion in the pancreatic ducts (Zaccagnino et al., 2016).

Kv10.1 is another Kv channel that has been reported in pancreatic cancer. The expression of Kv10.1 in peripheral tissues is very restricted (Hemmerlein et al., 2006), including pancreatic tissue (Pardo et al., 1999). A xenograft mouse model of pancreatic cancer showed that monoclonal antibodies blocking the Kv10.1 current exerts antitumor activity (Gomez-Varela et al., 2007). Because Kv10.1 is nearly absent in normal tissue, there is a certain tumor selectivity for Kv10.1 expression, which gives rise to the possibility that Kv10.1 can be used as a targeting channel for the delivery of cytotoxic compounds (Pardo and Stuhmer, 2014). However, the expression and function of Kv10.1 in PDAC must be further investigated.

Interestingly, another Kv channel, Kv11.1 has been implicated as an oncogene in various cancers, including PDAC (Arcangeli et al., 2014; Lastraioli et al., 2015a). In contrast, to the Kv10.1 expression, Kv11.1 is ubiquitously expressed in normal human tissues including heart where it is mainly expressed (Sanguinetti et al., 1995; McDonald et al., 1997; Pond et al., 2000; Camacho, 2006; Comes et al., 2015). *KCNH2* (gene coding for Kv11.1) was identified as a gene with somatic mutations that could drive the metastatic process of PDAC (Zhou et al., 2012). Here, exome sequencing analysis showed that *KCNH2* clustered into a single network related to cancer development. To investigate the involvement of *KCNH2* in PDAC progression, the authors showed that knockdown of Kv11.1 reduced proliferation, colony formation and migration in PDAC cell lines. Immunohistochemical analysis of Kv11.1 expression showed expression in 8 out of 38 (21%) PDAC tissues, versus one out of 37 (2.7%) in normal tissues (Zhou et al., 2012). Another study further investigated the expression and role of Kv11.1 in PDAC (Feng et al., 2014). Here, immunohistochemical analysis confirmed a strong expression in PDAC tissues, with highest expression in the cytoplasm and membrane. In contrary, normal tissue showed only weak expression. The expression was confirmed in PDAC cell lines (Feng et al., 2014). Knockdown

of Kv11.1 showed a significant decreased proliferation rate, higher number of cells undergoing apoptosis, cell cycle arrest in G1 phase and a reduction of migration and invasion, suggesting that Kv11.1 has a role in different aspects of PDAC progression (Feng et al., 2014). This was confirmed in a xenograft mouse model, were a knockdown of Kv11.1 in CFPAC-1 cells showed reduced tumor growth and fewer metastatic nodules, compared to tumors in mice injected with control cells (Feng et al., 2014). Furthermore, it was found that miR-96 was downregulated in tumor tissue and PDAC cells. The overexpression of miR-96 reduced cell proliferation, migration, and invasion *in-vitro* and reduced the Kv11.1 expression, tumor growth, and formation of metastasis *in-vivo* (Feng et al., 2014). This indicates that Kv11.1 could function as an oncogene in PDAC and be a potential target of miR-96 (Feng et al., 2014). Further investigations showed that Kv11.1 promotes pancreatic cancer cell migration, by modulation of F-actin organization and dynamics (Lastraioli et al., 2015b) suggesting its involvement in cancer metastasis (Arcangeli et al., 2014; Manoli et al., 2019).

KCa Channels/KCa3.1/IK

IK (KCa3.1) channels are the K^+ channels most frequently studied among this family in PDAC. Even though, transcripts of KCa4.1 and KCa4.2 also have been shown in some cell lines (Hayashi et al., 2012). Investigation of the KCa3.1 mRNA expression in primary pancreatic cancer tumors show that 8 of 9 tumors (89%) contain a 6- to 66-fold higher expression, compared to normal pancreatic tissue (Jager et al., 2004). KCa3.1 is also found overexpressed in several PDAC cell lines (Jager et al., 2004). The over-expression of KCa3.1 was associated with an increased Ca^{2+}-activated K^+-current. Pharmacological inhibition (by TRAM-34, Clotrimazole) of KCa3.1 completely suppressed cell proliferation of MiaPaCa-2 and BxPC-3 cells but not PANC-1 cells (Jager et al., 2004). Moreover, application of $[Ca^{2+}]_o$ while inhibiting with TRAM-34 or Clotrimazole rescued the MiaPaCa-2 and BxPC-3 cell proliferation but did not affect this of PANC-1 suggesting that PANC-1 cell line grows independently of functional KCa3.1 channels (Jager et al., 2004). Bonito and co-workers have also reported the role of KCa3.1 in PDAC cell proliferation and migration (Bonito et al., 2016). They showed a significant mRNA upregulation of KCa3.1 in MiaPaCa-2 and BxPC-3, but not in Capan-1 and PANC-1 cells. In addition, Patch clamp measurements revealed a Ca^{2+}-activated K^+ current, which was reduced by TRAM-34 and clotrimazole. Interestingly, a transient gene silencing of KCa3.1 in MiaPaCa-2 cells completely abolished the Ca^{2+} current (Bonito et al., 2016). MiaPaca-2 cell proliferation was inhibited with TRAM-34 and 1% FBS, whereas no effect was found by application of TRAM-34 and 10% FBS in the culture media, as shown before (Jager et al., 2004). Silencing of KCa3.1 removed the ability of MiaPaCa-2 cells to proliferate, and attenuated their cell invasion and migration. Surprisingly treatment upon TRAM-34 or clotrimazole increased cell migration. It was hypothesized that this could be due to Ca^{2+} homeostasis, which was investigated by Ca^{2+} imaging that confirmed that TRAM-34 evoked an increase of $[Ca^{2+}]_i$ (Bonito et al., 2016) possibly leading to promotion of cell migration (Lotz et al., 2004). This indicates that KCa3.1 expression and function are important for cell proliferation, migration and invasion (Bonito et al., 2016). Another study has identified KCa3.1 as a regulator of oxidative phosphorylation in MiaPaCa-2 cells as silencing and inhibition of KCa3.1 determined the effect of channel dependent-oxidative phosphorylation in proliferation and ATP generation (Kovalenko et al., 2016). In addition, MiaPaCa-2 cells showed mRNA and protein levels in mitochondria, suggesting that KCa3.1 is involved in proliferation through metabolic processes (Kovalenko et al., 2016). Three other studies have identified *KCNN4* (gene coding for KCa3.1) as a gene related to PDAC as its transcripts and gene-level were upregulated compared to normal pancreatic tissue (Zaccagnino et al., 2016; Jiang et al., 2017; Shen et al., 2017). The upregulation of *KCNN4* was associated with the gene expression of different EMT transcription factors (Zaccagnino et al., 2016).

Two-Pore K^+ Channels (K_2P)

The outward conducting, pH and membrane potential activated K_2P channels have an impact on physiological processes. They can regulate the cell volume, the membrane potential in form of being pH sensitive, modulate ion transport and Ca^{2+} homeostasis. They are involved in cancer progression due to their impact on cell growth survival and migration, as it has been shown in different types of cancer (Mu et al., 2003; Kim et al., 2004; Voloshyna et al., 2008; Alvarez-Baron et al., 2011; Lee G. W. et al., 2012; Nagy et al., 2014; Sauter et al., 2016). A broad data base analysis of K_2P expression in different cancers revealed an aberrant expression of different K_2P in PDAC (Williams et al., 2013). mRNA expression of *KCNK1* (gene coding for TWIK-1) was upregulated in PDAC compared to normal tissue, and *KCNK3* (gene coding for TASK-1) were downregulated (Williams et al., 2013). One study has found the functional mRNA and protein expression of *KCNK5* (gene coding for TASK-2) in PDAC cell lines HPAF, but the role in cancer progression was not further studied (Fong et al., 2003). In another study a pH sensitive K^+ current was identified in BxPC-3 cells and was probably mediated by TREK-1 (Sauter et al., 2016). TREK-1 protein expression was shown in PDAC cell lines where it was shown that TREK-1 was involved in proliferation (Sauter et al., 2016). A similar pattern was shown in a scratch wound healing assay were activation of TREK-1 lead to decreased migration. These results indicate that TREK-1 has a potential inhibiting role in PDAC proliferation and migration (Sauter et al., 2016). Very few studies have been done on the role of K_2P channels in PDAC. However, it can be suggested from other types of cancer that these channels can be related to cancer progression (Comes et al., 2015).

Calcium Channels in PDAC

ORAI and STIM

It is well known that physiological Ca^{2+} signaling has many effects in the exocrine pancreas, and takes part in stimulating secretion of HCO_3^- and other ions (Hegyi and Petersen, 2013; Maleth and Hegyi, 2014). In non-excitable cells, such as cancer cells, Ca^{2+} entry

occurs mainly through SOCs (Mo and Yang, 2018) but also through transient receptor potential channels (TRP), which are selective for both Ca^{2+} and Na^{+} (Worley et al., 2007).

There is increasing evidence of dysregulated Ca^{2+} signaling in cancer. This evidence is based on the implication of SOCs and TRP in key hallmarks of cancer progression and as prognostic markers in several types of cancers (Prevarskaya et al., 2007; Shapovalov et al., 2016; Chen et al., 2019). Some members of SOCs and TRP have been studied in PDAC, even though knowledge is less pronounced compared to other types of cancer, such as breast-, cervical-, and colorectal cancer (Chen et al., 2019).

The complex of ORAI1 and STIM1 has been shown to play a role in carcinogenesis and to be involved in regulation of proliferation, migration, invasion and apoptosis in different types of cancer (Chen et al., 2019). Only two studies have been performed on PDAC showing that ORAI1 and STIM1 mediate SOC entry and that they are involved in proliferation, survival and apoptosis (Kondratska et al., 2014; Khan et al., 2020). It has been shown that both ORAI1 and STIM1 were expressed in several PDAC cell lines at mRNA and protein levels, with PANC-1 showing the highest levels of both. Knockdown of ORAI1 and STIM1 with siRNA showed a significant reduction of Ca^{2+} entry. This was confirmed in PANC-1, AsPC-1, MiaPaCa-2, and Capan-1 cells, indicating that SOC entry is mediated by ORAI1 and STIM1 in different PDAC cell lines. A recent study has revealed the involvement of Calcium Release-Activated calcium (CRAC) channel (ORAI1) in proliferation of PDAC (Khan et al., 2020). An inhibition with CRAC channel inhibitor, RP4010, showed a significant reduction of cell proliferation and colony formation in MiaPaCa-2 cells and in L3.6pl (a pancreatic adenosquamous carcinoma derivated cell line). The influx of calcium was also inhibited upon treatment with RP4010, suggesting that cell proliferation is mediated by regulation of Ca^{2+} entry through CRAC channel (Khan et al., 2020). It was proposed that cell proliferation was calcium-regulated through the AKT/mTOR signaling pathway as RP4010 inhibition decreased the mRNA levels and protein expression of phosphorylated AKT, modulated the expression of proteins important for downstream AKT/mTOR signaling. Furthermore, RP4010 or ORAI1 knockdown showed a decrease in mRNA levels and in nuclear translocation of NFAT1, suggesting that CRAC channel takes part in modulating calcium signaling associated with NFAT translocation and that PDAC proliferation is regulated through the calcium-activated AKT/mTOR/NFAT signaling (Khan et al., 2020). To test if RP4010 could enhance anticancer activity of standard used treatments gemcitabine and Nab-Paclitaxel, a combination of the three drugs were used to treat PDAC cell lines. The results showed a decrease in proliferation. A synergistic effect of certain dose combinations of RP4010 with gemcitabine/Nab-Paclitaxel was found to inhibit cell growth. In addition, this synergistic treatment downregulated the expression of NFATC1 and mTOR mRNA and NFAT1, NF-κB, and phosphorylated S6K proteins, suggesting that inhibition of cell proliferation through CRAC channel are mediated by a downregulation of mTOR, NFAT and NF-κB signaling (Khan et al., 2020). The anticancer activity and the synergistic effect of RP4010/Gemcitabine/Nab-Paclitaxel were tested *in-vivo*. In a patient-derived xenograft mouse model, it was shown that Ki-67 expression decreased with the treatment of RP4010 or by the triple combination treatment (Khan et al., 2020). The overexpression found by Kondratska and co-workers can explain increased $[Ca^{2+}]_i$ levels in PDAC cell lines, and that this is a mechanism for survival (Kondratska et al., 2014). In contrast, another study has found decreased gene expression levels of ORAI1 (Zaccagnino et al., 2016).

A recent study has been investigating the role of STIM1 in PDAC progression (Wang et al., 2019). shRNA knockdown of STIM1 showed decreased proliferation, invasion, and upregulation of E-cadherin protein levels and downregulation of vimentin levels, suggesting that STIM1 is involved in carcinogenesis of PDAC and in some way involved in Epithelial-Mesenchymal transition (EMT). Even though, E-cadherin levels have shown to be upregulated, in contrary to what is usually seen in cells undergoing EMT where E-cadherin decrease in favor of N-cadherin (Gheldof and Berx, 2013). Furthermore, tissue microarray analysis showed that the STIM1 expression positively correlated with HIF-1α (Wang et al., 2019). It was further shown that similar protein expression levels of STIM1 and HIF-1α were expressed in different PDAC cell lines. STIM1 and HIF-1α protein levels were also upregulated in some PDAC tumor samples compared to non-tumor samples. Knockdown of HIF-1α in PANC-1 cells revealed a significantly lower mRNA and protein expression of STIM1. The co-upregulation of both proteins and the downregulation of STIM1 upon knockdown of HIF-1α indicate that STIM1 is regulated by HIF-1α on the transcriptional level. STIM1 promoter activity was tested in PANC-1 cells upon normoxia or hypoxia, where HIF-1α binding sites, under hypoxic conditions reduced STIM1 promoter activity (Wang et al., 2019). These results indicate that HIF-1α probably regulates STIM1 transcription and that STIM1 overexpression, in a hypoxic environment, can promote PDAC progression and invasion (Wang et al., 2019).

The EMT process is stimulated upon loss of cell-cell contact and occurs in migrating cancer cells (Gheldof and Berx, 2013). It has been shown in disconnected individual PANC-1 cells that ER/Plasma membrane junctions containing STIM1, together with the IP_3Rs, redistribute to the leading edge of focal adhesions (Okeke et al., 2016). An inhibition of IP_3Rs and SOC entry reduced the migrating capacity of PANC-1 cells. This mechanism indicates the importance of Ca^{2+} signaling in migration through SOC entry and intracellular calcium channels (Okeke et al., 2016).

TRP Channels

TRP form an adaptable family of ion channel proteins where the majority are calcium permeable and show regulatory patterns that are sensitive to different environmental factors (Shapovalov et al., 2016). The role of TRP has been reported in different types of cancer (Prevarskaya et al., 2007). It has been proposed that

TRPC1 can regulate PDAC cell proliferation through TGF-β signaling, as TGF-β has been shown to be one of the key modulators of EMT in mammary epithelial cells (Radisky and LaBarge, 2008). In PDAC cell line BxPC-3, TGF-β has shown to induce $[Ca^{2+}]_i$ increase leading to activation of the Ca^{2+}-dependent protein kinase Cα (PKC-α) and its translocation to the plasma membrane. PKC-α activation by TGF-β initiates the motility and migration by inhibiting tumor suppressor PTEN (Chow et al., 2008). Further on, it has been shown that there is a high expression of TRPC1, TRPC4 and TRPC6 in BxPC-3 cells (Dong et al., 2010). Here, it was confirmed that TGF-β induces cytosolic Ca^{2+} concentrations through TRPC1, followed by a PKC-α activation, thus initiating motility and migration. This was shown by a pharmacological inhibition of SOC entry pathways with 2-APB and La^{3+}, which abolishes the TGF-β induced cytosolic Ca^{2+} increase. Furthermore, blocking of PKC-α with selective PKC-α inhibitors inhibited the TGF-β mediated Ca^{2+} entry. In addition, knockdown of TRPC1 with siRNA reversed the effect of TGF-β on cell motility, although, knockdown of TRPC4 and TRPC6 did not have an effect on motility of TGF-β mediated BxPC-3 cell motility (Dong et al., 2010). These observations suggest that dysregulated Ca^{2+} entry through TRPC1 could be involved in EMT, and thereby invasion and metastasis of PDAC.

TRPV channels function as sensors in the central and peripheral nervous system where the majority is sensitive to voltage and temperature (Premkumar and Abooj, 2013). TRPV1 has shown to be related to oncogenesis and is expressed in different types of cancer (Domotor et al., 2005; Lazzeri et al., 2005; Sanchez et al., 2005; Miao et al., 2008; Morelli et al., 2014; Vercelli et al., 2014). TRPV1 can be activated by multiple pathways, which can promote pancreatic inflammation and pain, but also pancreatic cancer (Hartel et al., 2006; Huang et al., 2020). TRPV1 was shown to be upregulated at the mRNA and protein level in PDAC tissue compared to normal pancreatic tissue (Hartel et al., 2006). TRPV1 staining has been shown in both normal acini and ducts but with highest intensity in nerves of inflamed tissue surrounding the cancer. The elevated TRPV1 expression in infiltrating nerves was associated with pain in patients with PDAC. The same authors showed that inhibition of TRPV1 with resiniferatoxin induces apoptosis by targeting mitochondrial respiration and decreases cell growth in some PDAC cell lines (Hartel et al., 2006).

Recently, it has been shown that TRPV1 regulates the Epidermal Growth Factor Receptor (EGFR) in PANC-1 cell line (Huang et al., 2020). In this study, an overexpression of TRPV1 has been associated with a decrease in protein expression of EGFR in PANC-1. Vice versa, the downregulation and inhibition of TRPV1 increases the protein expression of EGFR. In addition, an overexpression of TRPV1 showed increased levels of ubiquitinated EGFR. The membranous fractions of EGFR were reduced, while the cytoplasmic were increased compared to the control (Huang et al., 2020). This indicates that TRPV1 promotes EGFR ubiquitination and thereby a downregulation of EGFR activity, resulting in EGFR cytoplasmic translocation and degradation, which was found to be mainly through the lysosomal pathway (Huang et al., 2020). Furthermore, it was shown that TRPV1 overexpression inhibited proliferation, probably through the MAPK signaling pathway. Overexpression of TRPV1 resulted in decreased mRNA levels of KRAS and AKT2 and a treatment with EGF reduced the protein expression of ERK, JNK, and CREB, suggesting that a TRPV1 overexpression decreases EGFR/MAPK dependent proliferation in PANC-1 cells (Huang et al., 2020). The two above mentioned studies show contrary results in form of how the expression of TRPV1 is related to proliferation. Hartel et al., demonstrated that inhibition of TRPV1 terminate cell growth and induced apoptosis, where Huang et al., found that an overexpression of TRPV1 leads to a reduced proliferation rate (Hartel et al., 2006; Huang et al., 2020).

Another member of the TRPV family, TRPV6, was also found to be overexpressed in the primary pancreatic cancer tissues at both protein and mRNA levels. Moreover, by immunohistochemical analysis, it was found that TRPV6 is mainly localized in the cytoplasm in both tumor and normal tissue (Song et al., 2018). *In-vitro*, the highest level of TRPV6 was found in two pancreatic cell lines, Capan-2 and SW1990. The knockdown of TRPV6, by siRNA, resulted in reduced proliferation, cell cycle arrest in G0/G1 phase, promotion of apoptosis, and suppression of cell migration and invasion (Song et al., 2018). Furthermore, the silencing of TRPV6 resulted in a significant increase of sensitivity to the chemotherapeutic reagent oxaliplatin (Song et al., 2018). In contrast to this finding, Zaccagnino et al., 2016 showed a downregulation of TRPV6 in PDAC tissue, compared to normal pancreatic epithelium. Moreover, Tawfik et al., 2020 found also a downregulation of TRPV6 expression in a PDAC cell line A818–6 grown in a highly malignant undifferentiated monolayer. The authors suggest that a lower expression of TRPV6 could contribute to an inhibited epithelial fluid secretion in PDAC (Tawfik et al., 2020).

The TRPM family is also constituted with several members, which have been found to be implicated in carcinogenesis. One of the most studied in PDAC is TRPM7, which is a particular channel having an intrinsic kinase, together with its closest homolog TRPM6 (Yee et al., 2012a). TRMP7 is ubiquitously expressed and controls cellular homeostasis of ions, especially Mg^{2+} and Ca^{2+}. Interpreting that the developmental role of TRPM7 in zebrafish could be the same in humans, the role of TRPM7 has been studied in the development and progression of PDAC. Here, it has been shown that there was an overexpression of TRPM7 protein in PDAC tissue compared to normal tissue, and that TRPM7 is required for Mg^{2+}-regulated proliferation. Knockdown of TRPM7 with siRNA showed that this channel is necessary to prevent cell cycle arrest in G0/G1 phases. Furthermore, the proliferation of TRPM7-deficient PDAC cells was rescued by adding Mg^{2+} to the cell culture medium (Yee et al., 2011). Another study confirmed the overexpression of TRPM7 both at mRNA and protein levels in PDAC tissue (Rybarczyk et al., 2012). Furthermore, it was shown that TRPM7 silenced BxPC-3 cells decreased $[Mg^{2+}]_i$, suggesting that TRPM7 takes part in regulating Mg^{2+} uptake in PDAC cells. In contrary to previous findings, these authors demonstrated that the

silencing of TRPM7 had no effect on cell viability or proliferation, but a significant decrease of BxPC-3 cell migration (Rybarczyk et al., 2012). TRPM7 has also been found to be involved in cell invasion in both MiaPaCa2- and PANC-1 cells. In the two last cell lines, TRPM7 regulates constitutive cation currents, the influx and homeostasis of Mg^{2+}, and cell invasion through the Hsp90α/uPA/MMP-2 proteolytic pathway (Rybarczyk et al., 2017).

Besides TRPM7, also other TRPM channels are found to be expressed in pancreatic cancer (Yee et al., 2010; Yee et al., 2014). TRPM8 is expressed in different types of adult human tissue and has also been found to be expressed in PDAC. In a panel of PDAC cell lines, mRNA TRPM8 was consistently overexpressed compared to the control cell line (H6c7) (Yee et al., 2010). This pattern has further been confirmed by immunohistochemistry in human PDAC tumors, compared to normal pancreatic tissue (Yee et al., 2010; Yee et al., 2014). Here, it was found that TRPM8 has a role in carcinogenesis in form of proliferation, migration and senescence. TRPM8 is required for proliferation by promoting cell cycle progression in PANC-1 and BxPC-3 cells, as a knockdown of TRPM8 showed a significant decrease in proliferation rate and a cell cycle arrest in G0/G1 phase (Yee et al., 2010). In another study, the knockdown of TRPM8 showed the opposite effect on proliferation. Here, the proliferation increased by 30% in PANC-1 cells and in contrary the proliferation was suppressed in HEK/M8 cells. It was found that TRPM8 is expressed in a non-glycosylated form in different PDAC cell lines, and that the channel in this form might have a protective role in PDAC (Ulareanu et al., 2017). Concerning the involvement of TRPM8 in migration and invasion, two studies show opposite results. One study demonstrated that TRPM8 also is required for cell migration, as a knockdown of the channel impaired migration of BxPC-3 and MiaPaCa-2 by 60% and 45%, respectively (Yee et al., 2014). Where another study found that it enhanced the motility of PANC-1 cells (Cucu et al., 2014).

Recently, it has been shown that a third member of the TRPM family, also plays a role in PDAC progression (Lin et al., 2018). An overexpression of TRPM2 enhanced the proliferative, migrative and invasive abilities of PANC-1 cells, compared to the control cells and the results were inversed when TRPM2 was silenced in PANC-1 cells. It should be noted, that the study does not mention the application of a proliferation inhibitor during the Scratch wound-healing assay, which investigates the migratory role of TRPM2. Therefore, one can speculate if the wound-healing could be caused by proliferation, and not migration. Nevertheless, these results suggest that TRPM2 is involved at least in cell growth and invasion (Lin et al., 2018).

Voltage-Dependent Calcium Channels

The expression of two voltage dependent Ca^{2+} channels have been found to be dysregulated in PDAC, namely, CaV2.1 (*CACNA1A*) and CaV3.1 (*CACNA1G*) are upregulated and downregulated, respectively (Zaccagnino et al., 2016). These sparse data indicate that voltage-dependent Ca^{2+} channels might have a role in PDAC progression.

Chloride Channels in PDAC

Ca^{2+}-Activated Chloride Channel (CaCC) and TMEM Proteins

Aberrant expression and dysregulated function of Cl^- channels have shown to be involved in carcinogenesis, especially their role in cell volume regulation has shown to be important for cancer cell migration and infiltration (Duran et al., 2010; Prevarskaya et al., 2010; Anderson et al., 2019). In Capan-1 cells, CaCC are expressed at the apical membrane, as shown for normal pancreatic acinar and ductal cells (Park et al., 2001; Wang et al., 2013; Wang and Novak, 2013).

The functional role of TMEM16A has been found to vary between different types of cancer (Ayoub et al., 2010; Liu et al., 2012; Ruiz et al., 2012; Britschgi et al., 2013). While a pro-proliferative role was found in breast and prostate cancer, the role in pancreatic cancer has been found to be contradictory. An anti-proliferative effect was found by a knockdown and overexpression strategy in CFPAC-1 cells (Ruiz et al., 2012), where another study found that inhibition with the TMEM16A specific inhibitor $T16A_{inh}$-A01 decreased the proliferation rate in CFPAC-1 cells (Mazzone et al., 2012). Both studies lack the comparison of PDAC cell lines with a normal pancreatic epithelial control cell line. This was considered in a recent study, where the role of TMEM16A was investigated in PDAC cell lines and compared to a normal pancreatic epithelial control cell line (Sauter et al., 2015). The mRNA expression of TMEM16A was upregulated, with a 1,450-fold, in AsPC-1, BxPC-3, and especially in Capan-1 cells (Sauter et al., 2015). The upregulation was confirmed by an increase in TMEM16A protein expression for all three cell lines. Furthermore, it was shown that TMEM16A carries the major component of CaCC current in these cell lines (Sauter et al., 2015). Moreover, the authors found that knockdown of TMEM16A had no effect on proliferation. Inhibition, by $T16A_{inh}$-A01 or other CaCC inhibitors, failed to affect PDAC cell lines proliferation, while $T16A_{inh}$-A01 had a significant effect on the control cell line cell proliferation, which almost completely lack the expression of TMEM16A. These results suggest that the inhibition by $T16A_{inh}$-A01 is unspecific for TMEM16A, and that this channel has no implication in proliferation, at least in these three PDAC cell lines (Sauter et al., 2015). According to the role of TMEM16A in migration, gene silencing reduced the migratory capability of AsPC-1 and BxPC-3 cells, where the inhibition with $T16A_{inh}$-A01 was ineffective (Sauter et al., 2015). Other CaCC inhibitors caused a decrease in migration of BxPC-3 cells. Nevertheless, Capan-1 cells showed the highest expression of TMEM16A, the migration was very slow, suggesting that TMEM16A is not implicated in the role of migration in Capan-1 cells and supporting that TMEM16A has different roles in carcinogenesis of PDAC cells (Sauter et al., 2015).

Another recent study has performed a database investigation on the expression of TMEM16A and found that mRNA TMEM16A expression is upregulated in pancreatic cancer (Crottes et al., 2019). The authors found that extracellular application of EGF increased $[Ca^{2+}]_i$ and the outward-rectifying Cl^- current, which were both inhibited by different

TMEM16A inhibitors. The regulation of Cl^- currents and the Ca^{2+} response were probably due to SOC entry. Furthermore, silencing of TMEM16A in AsPC-1 cells reduced migration even under EGF treatments, while EGF induced migration in the control cell line. This indicates that TMEM16A is involved in EGF-induced PDAC migration and progression, probably through Ca^{2+} signaling. In addition, this study investigated the possible role of TMEM16A to classify PDAC patients (Crottes et al., 2019). They found 10 genes involved in EGF-induced TMEM16A-dependent Ca^{2+} signaling, which could distinguish neuro-endocrine tumors from other pancreatic cancers. In PDAC, these genes formed three clusters with different genetic profiles that could reflect different molecular characterizations (Crottes et al., 2019).

Another TMEM16 protein expressed in pancreatic cancer is the TMEM16J protein, which also has been found to be overexpressed (Jun et al., 2017). TMEM16J is not a well characterized protein, but it is proposed that it might function as a cation channel activated by the cAMP/PKA signaling pathway (Falzone et al., 2018; Kim et al., 2018). An upregulation of TMEM16J gene-, mRNA-, and protein overexpression were found in AsPC-1, BxPC-3, and Capan-2 cell lines and a small overexpression in PANC-1 cells. An overexpression of TMEM16J in PANC-1 cells resulted in phosphorylated ERK1/2 levels, but not total ERK1/2 levels. Furthermore, both EGFR and phosphorylated EGFR levels were upregulated in PANC-1 cells overexpressing TMEM16J and an immunoprecipitation assay revealed that both TMEM16A and TMEM16J formed protein complexes with EGFR, but the binding affinity for TMEM16J was 132% higher, than for the one of TMEM16A (Jun et al., 2017), suggesting that TMEM16J are involved in upregulation and activation of EGFR. In contrary, a knockdown of TMEM16J in AsPC-1 cells resulted in inhibition of phosphorylated ERK1/2, EGFR and phosphorylated EGFR and a decreased proliferation rate. These results were confirmed *in-vivo*, were a xenograft mouse model was made by implanting PANC-1 cells stably overexpressing TMEM16J. It was shown that tumor growth was significantly increased and immunohistochemistry of these tumors confirmed the TMEM16J overexpression (Jun et al., 2017). These results indicate that TMEM16J is implicated in cell proliferation and tumor growth. Another member of the TMEM16 family, TMEM16E, has been shown to be implicated in PDAC. It is not yet clear whether the TMEM16E protein function as an ion channel or scramblase (Falzone et al., 2018). It has been shown, by immunohistochemical analysis, that TMEM16E is entirely expressed in PDAC but not in normal pancreatic tissue (Song et al., 2019). The highest expression of both mRNA and protein of TMEM16E was found in PANC-1 cells. The impact of TMEM16E on migration was investigated by a wound-scratch assay and a siRNA knockdown of TMEM16E showed a significant decrease in PANC-1 cell migration (Song et al., 2019). Even though, it should be mentioned that the authors do not account for the possible effect of proliferation in this assay. The migration was in some ways confirmed by the downregulation of vimentin protein expression, compared to the control, which showed a higher expression of vimentin, suggesting that TMEM16E is implicated in migration of PANC-1 cells. In addition, the proliferation of PANC-1 cells was significantly decreased upon knockdown of TMEM16E suggesting its role in proliferation (Song et al., 2019). This assay supports the speculation on the TMEM16E role in migration.

Besides being activated by Ca^{2+}, CaCC can also be activated and regulated by specific proteins, namely Calcium-activated Chloride channel regulators (CLCAs) also called Calcium Chloride channel accessory proteins. CLCAs are expressed in different types of cancer and have been implicated in regulation of proliferation, migration and metastasis (Yurtsever et al., 2012; Lang and Stournaras, 2014; Stock and Schwab, 2015). CLCA1 has been shown to be overexpressed in pancreatic cancer (Hu et al., 2018a; Hu et al., 2018b). However, the expression pattern and underlying molecular mechanism of its role in PDAC is less known. Finally, low gene expression of Chloride Channel Kb (*CLCNKB*) and Chloride Voltage-Gated Channel 1 (*CLCN1)* have been reported in human PDAC tissue compared to normal pancreatic epithelium (Zaccagnino et al., 2016).

CFTR

It has been shown that CFTR is expressed in some PDAC cell lines. An early study showed that CFTR only was expressed in Capan-1 cells among nine different pancreatic cell lines and that the expression varied as a function of confluence (Chambers and Harris, 1993). Singh et al., confirmed the almost non-existent expression of CFTR in PDAC cell lines. Indeed, mRNA levels were detectable in normal pancreatic tissue and three (Capan-1, Suit2 and SW1990) out of 16 pancreatic cell lines (Singh et al., 2007). Furthermore, Zaccagnino et al., reported the downregulation of CFTR, at gene-level, in human PDAC tissue compared to normal pancreatic epithelium. This downregulation was associated with gene expression of EMT transcription factors (Zaccagnino et al., 2016). Furthermore, Singh et al., showed that wild type CFTR negatively regulated MUC4 expression while silencing of CFTR upregulated MUC4 expression. As MUC4 is a protein involved in tumor migration and metastasis, the negative regulation by CFTR indicates a protective role and a tumor suppressing function by inhibiting MUC4 and hence pancreatic cancer progression (Singh et al., 2007).

A recent study has investigated CFTR expression in patient derivated PDAC organoids, in order to enable routine organoid subtyping for personalized treatment (Hennig et al., 2019). It has been suggested that subtyping could be based on the expression of cytokeratin 81 (KRT81) and hepatocyte nuclear factor 1A (HNF1A). As the antibody for HNF1A was no longer available, the authors permitted CFTR to replace it as a potential marker instead of HNF1A (Hennig et al., 2019). Organoids can be categorized into the established quasi-mesenchymal, exocrine-like, and classical subtypes. Immunofluorescence staining showed a mutual expression pattern where exocrine-like organoids were $CFTR^+/KRT81^-$ and quasi-mesenchymal $CFTR^-/KRT81^+$. The protein expression revealed by IF was compared to mRNA levels of CFTR, which matched in 8 out of 10 cases (Hennig et al., 2019). In addition, it was confirmed, by immunohistochemical analysis, that both CFTR and KRT81

were preserved in 6 out of 7 tumors, indicating that the organoids had the same subtype as their primary tumor (Hennig et al., 2019). These results suggest that CFTR could be a supplement marker for HFN1A and that CFTR/KRT81 together might be a suitable way to evaluate subtype organoids for personal treatments (Hennig et al., 2019).

Cl^- Intracellular Channel Proteins (CLICs)

CLICs are ubiquitously expressed and have been identified in several types of cancer, where they are either overexpressed or downregulated compared to the normal tissue (Peretti et al., 2015). In PDAC, CLICs are mostly found upregulated, even though their specific role in PDAC progression and development is not yet understood. CLIC2, CLIC3, and CLIC5 have been shown to be expressed at mRNA and protein levels in a HPAF cell line. By an electrophysiological study, the authors revealed that there was no single channel/conductance for apical Cl^- secretion, but that these CLICs rather contributed to provide a constant net conductance across the plasma membrane (Fong et al., 2003). Another study has shown the importance of CLIC3 in PDAC, as immunohistochemical analysis and mRNA levels showed an overexpression of CLIC3 in PDAC tissue compared to normal pancreatic tissue (Dozynkiewicz et al., 2012). It was also found that CLIC3 in collaboration with Rab25 promoted cancer cell invasion and migration by integrin recycling from late endosomes/lysosomes (Dozynkiewicz et al., 2012). CLIC1 was overexpressed in primary tumors compared to normal pancreatic tissue, and strongly expressed in MiaPaCa-2 and PANC-1 cells (Lu et al., 2015). Silencing of CLIC1 showed a significant decrease in the proliferation rate, colony formation and the invasive abilities of both MiaPaCa-2 and PANC-1 cells, suggesting that CLIC1 contributes to the aggressive role of these PDAC cells (Lu et al., 2015). In addition, gene expression levels of CLIC5 has been found to be downregulated in PDAC tissue, compared to normal pancreatic epithelium and to be associated with gene expression of transcription factors related to cell differentiation (Zaccagnino et al., 2016).

Aquaporins (AQPs) in PDAC

AQPs are expressed in various types of cancers and are predicted to be key regulators in tumor development and progression (Papadopoulos and Saadoun, 2015). The expression and role of AQPs in PDAC is poorly studied, yet few studies have described their involvement in PDAC progression (Burghardt et al., 2003; Direito et al., 2017; Huang et al., 2017; Zou et al., 2019). Burghardt and co-workers have found mRNA expression of AQP1, AQP3, AQP4, AQP5, and AQP8 in PDAC. All subtypes were expressed in solid tumors, where only AQP3, AQP4, and AQP5 were expressed in PDAC cell lines (Burghardt et al., 2003). Further studies have found an upregulation of AQP1 and AQP3 in PDAC tissue compared to normal pancreatic tissue (Zou et al., 2019). The expression of AQP1 correlated with the expression of AQP3, suggesting that these two channels cooperate during PDAC development. Another study has shown the overexpression of AQP3 and AQP5 in PDAC tissue (Direito et al., 2017). AQP5 localization in PDAC was found to be in the entire plasma membrane and in the cytoplasm of ducts cells, where in normal pancreas the localization is in the apical membrane. Furthermore, AQP5 and AQP3 were suggested to be involved in proliferation and tumor transformation as a simultaneous overexpression was found to be correlated with an increased expression of EGFR, Ki-67, CK7, and a decrease of E-cadherin and increase of Vimentin (Direito et al., 2017). Another study investigating AQP has, through TCGA analysis, revealed that AQP3 shows the highest expression among AQPs in PDAC (Huang et al., 2017). The authors investigated the role of AQP3 further, with a focus on how microRNA (miR-874) regulates gene expression and post-translational events in PDAC. In a panel of eight pancreatic cell lines, they detected that cell lines with high AQP3 mRNA levels had lower miR-874 levels, where cell lines with high miR-874 had lower AQP3 levels suggesting that AQP3 expression is regulated by miR-874 (Huang et al., 2017). It was found that both modulation of AQP3 and miR-874 altered the expression and activity of mTOR and its downstream target S6, suggesting that an overexpression of AQP3 is associated with proliferation and cell survival by mTOR signaling in PDAC. In contrast to other studies, Zaccagnino et al. (2016) showed a downregulation of AQP3 and AQP8 expression in PDAC tissue compared to normal pancreatic one. Furthermore, they showed that AQP3 expression was associated with several cell differentiation related transcription factors.

Sodium Channels in PDAC

ASIC

It has recently been shown that acid-sensing ion channels (ASICs), an H^+-gated subgroup of ENaC, are expressed in PDAC cell lines and tissue (Zhu et al., 2017). ASIC1 and ASIC3 were found functionally expressed and mRNA and protein expression were also found in PDAC cell lines. In all cases, the expression was upregulated compared to the normal control cell line. These results were confirmed in PDAC tissue where immunohistochemical analysis and qPCR revealed the overexpression compared to non-cancerous pancreatic tissue, suggesting that ASIC1 and ASIC3 have a pathophysiological role in PDAC (Zhu et al., 2017). Separate inhibition or knockdown of ASIC1 and ASIC3 decreased the acidity-promoted invasion and migration capacity of PDAC cell lines, but did not decrease the proliferation rate, suggesting that ASIC1 and ASIC3 are involved in the metastatic process of PDAC, but not tumor cell growth (Zhu et al., 2017). Furthermore, it was shown that ASIC1 and ASIC3 are involved in acidity-promoted EMT, as silencing or inhibition of ASIC1 or ASIC3 in PDAC cells showed decreased protein expression of mesenchymal markers Vimentin, N-cadherin, Snail, and ZEB1, while the epithelial marker E-cadherin showed increased protein expression. In contrary, PDAC cells overexpressing ASIC1 and ASIC3 showed an increase in mesenchymal markers and a decrease in epithelial markers, under acidic conditions. This was confirmed in human PDAC tissue samples by IF analysis. It was further investigated whether this mechanism was regulated by $[Ca^{2+}]_i$, where it was found that inhibition of ASIC1 or ASIC3 resulted in a decrease of $[Ca^{2+}]_i$ upon acidification. In addition, the removal of $[Ca^{2+}]_i$ upon acidic conditions decreased mesenchymal markers and

increased the epithelial ones. It was determined that the RhoA pathway, which is involved in cytoskeleton re-arrangement and cell migration, was a major effector of EMT induced by ASIC1/3-$[Ca^{2+}]_i$ activation in acidic conditions (Zhu et al., 2017). The role of ASIC1 and ASIC3 was further confirmed *in-vivo*, where a xenograft mouse model injected with BxPC-3 cells with a stable knockdown of ASIC1 and ASIC3 showed a significant decrease in lung and liver metastasis, but no obvious effect on tumor growth (Zhu et al., 2017).

VGSCs

Another subfamily of Na^+ channels, namely voltage gated sodium channels (VGSCs), has shown to be implicated in cancer progression (Angus and Ruben, 2019). An early study has shown that Ca^{2+} blockers Phenytoin and Verapamil inhibited the growth of pancreatic cancer cell lines MiaPaCa-2 and CAV, both *in-vitro* and *in-vivo* (Sato et al., 1994). Phenytoin and Verapamil were chosen because they appeared to be blocking different Ca^{2+} channels; T-type and L-type voltage dependent Ca^{2+} channels, respectively (Sato et al., 1994). It has been suggested that this growth-inhibition of pancreatic cancer cell was rather due to the block of VGSC than the block of Ca^{2+} channels, as both Phenytoin and Verapamil show high affinity for VGSC in the inactivated state of the channel (Ragsdale et al., 1991; Koltai, 2015). In addition, the expression of VGSC (*SCN9A* and *SCN3A*) was downregulated in PDAC (Zaccagnino et al., 2016).

IONOTROPIC RECEPTORS IN PDAC

Purinergic Receptors (P2XR) and N-Methyl-D-Aspartate Receptors (NMDAR)

Different types of ionotropic receptors including P2XR and NMDAR have been reported to be expressed in PDAC (Kunzli et al., 2007; Hansen et al., 2008; Burnstock and Novak, 2012; Li and Hanahan, 2013; North et al., 2017). Among P2XR, P2X7R is the most well described (Kunzli et al., 2007; Hansen et al., 2008; Burnstock and Novak, 2012).This ionotropic receptor has shown to be overexpressed in PDAC cell lines and tissue (Kunzli et al., 2007; Giannuzzo et al., 2015), and to be implicated in the proliferating, apoptotic, migrating, and invading processes of PDAC (Kunzli et al., 2007; Hansen et al., 2008; Giannuzzo et al., 2015; Giannuzzo et al., 2016; Choi et al., 2018). In addition, the expression of NMDAR was found in both PDAC cell lines and PDAC tumors, and their inhibition and blocking resulted in reduced different PDAC cell lines viability and survival (Li and Hanahan, 2013; North et al., 2017). Furthermore, an inhibition of NMDAR prevented growth of tumor xenografts (Li and Hanahan, 2013; North et al., 2017).

ION CHANNELS AS PDAC BIOMARKERS

A growing number of studies have investigated ion channel expression in pancreatic cell lines and human tissues, showing modulation of mRNA and/or protein expression between normal and cancer cells. Among all the studied channels, only CFTR has lower expression in cancer cell lines compared to normal cells (Singh et al., 2007), while Kv1.3, Kv7.1, and TASK-1 were downregulated in PDAC tissue compared to healthy tissue (Brevet et al., 2009; Williams et al., 2013; Tawfik et al., 2020), suggesting a protective role and tumor suppressive function for these channels. Although most of the ion channels are overexpressed in PDAC, studies on ion channel expression patterns in correlation with clinical parameters are more limited.

Diagnostic Markers

Some attention has been given to the connection between pancreatic cancer risk and CFTR deficiency. Mutations in the *CFTR* gene cause the hereditary life shortening disease cystic fibrosis (CF). Severe clinical manifestations occur upon CF in secretory epithelial tissues and in pancreas, mutations causing loss of function lead to pancreatic insufficiency (Wilschanski and Novak, 2013; Castellani and Assael, 2017). Different cohort studies have investigated how different variants of *CFTR* affect the risk of pancreatic cancer (Sheldon et al., 1993; Neglia et al., 1995). It has been shown that CF patients present an elevated risk to develop pancreatic cancer, even though the overall risk of developing cancer is the same as for the general population (Sheldon et al., 1993; Neglia et al., 1995). Furthermore, studies also indicate that patients who are *CFTR* mutant carriers develop pancreatic cancer in a younger age, compared to patients carrying a wildtype form of *CFTR* (McWilliams et al., 2010; Hamoir et al., 2013), and patients carrying a germline mutation to some degree have an increased risk of developing PDAC (Cazacu et al., 2018). One mechanism of which a *CFTR* mutation could cause pancreatic cancer is by the defect of CFTR and ion transport leading to dysregulated mucus secretion and obstruction of the pancreatic ducts, which all are events that could result in pancreatitis (McWilliams et al., 2010). Patients with chronic pancreatitis have a 26-fold higher risk for developing pancreatic cancer compared to the general population (Lowenfels et al., 1993; Kirkegard et al., 2017), suggesting that *CFTR* mutation could be considered as a new risk factor for developing PDAC.

In order to discriminate pancreatic premalignant/malignant lesions from benign lesions, an explorative proteomic approach was performed on a cohort of 24 patients using targeting mass spectrometry analysis of different biomarkers (Jabbar et al., 2018). This study proposed CLCA1 to be a supportive marker, which together with mucin-5AC (MUC5AC) and prostate stem-cell antigen (PSCA) could distinguish cystic precursor lesions from PDAC, suggesting that CLCA1 is a potential biomarker in PDAC diagnosis.

Prognostic Markers of Cancer Progression and Aggressiveness

Different studies have investigated ion channels as potential biomarkers of PDAC development and progression. Using immunohistochemical analysis, high KCa3.1 expression in PDAC tissue was correlated with TNM stages III and IV

(Jiang et al., 2017), and high expression of STIM1 was correlated with tumor grade (Wang et al., 2019). Upregulation of Kv11.1 expression was associated with advanced tumor grade and high expression of Ki67 proliferative marker (Lastraioli et al., 2015b), whereas TRPV6, TRPM8 and AQP1/AQP3 channels were positively correlated with tumor stages III and IV and large tumor size (Yee et al., 2014; Du et al., 2018; Liu et al., 2018; Song et al., 2018; Zou et al., 2019). Finally, TRPM7 and Cl^- intracellular channel proteins (CLIC1-3). CLIC1 overexpression was shown to be correlated with the three clinical parameters: advanced tumor grade, advanced tumor stage and large tumor size (Rybarczyk et al., 2012; Lu et al., 2015; Yee et al., 2015; Jia et al., 2016). These results suggest that all the ion channels cited above are associated with pancreatic tumor growth.

Regarding the metastatic status, immunohistochemistry experiments showed that TRPV6 expression was higher in cases where PDAC was infiltrating (Song et al., 2018). The same results were observed at mRNA and protein levels for CLIC3, with a highly detectable expression in regions where the tumor was invading normal pancreatic tissue (Dozynkiewicz et al., 2012). Other studies revealed higher TRPM7 and TRPM8 staining in metastatic tumors than in non-metastatic tumors (Yee et al., 2015; Liu et al., 2018), which was confirmed by qPCR for TRPM8 (Du et al., 2018). AQP1 and AQP3 were also more expressed in PDAC patients with lymph node metastasis and invasion, than in non-invasive cancers (Zou et al., 2019), whereas overexpression of Kir3.1 potassium channel (GIRK1) was not found to be correlated with metastatic status (Brevet et al., 2009).

Gene expression correlation analysis demonstrated that TRPM2 is strongly correlated with different genes including toll-like receptor 7 (TLR7) (Lin et al., 2018), which has already been associated with PDAC progression (Ochi et al., 2012; Grimmig et al., 2015; Wang et al., 2016). Moreover, AQP1 and AQP3 protein expression was highest in poorly differentiated tumors (Direito et al., 2017; Zou et al., 2019), whereas AQP5 is more expressed in moderately differentiated tumors (Direito et al., 2017), suggesting that AQPs are associated with tumor aggressiveness.

Finally, most of the studied ion channels in PDAC tissue were associated with overall survival of the patients. The authors usually used immunohistochemical staining on large cohorts and Kaplan-Meier survival analysis, to observe a correlation between high channel expression and short patient survival. This is the case for KCa3.1 (Jiang et al., 2017), STIM1 (Wang et al., 2019), TRPM8 (Liu et al., 2018), TRPV6 (Song et al., 2018), TMEM16J (Jun et al., 2017), CLIC1 (Lu et al., 2015; Jia et al., 2016), and AQP1/AQP3 (Zou et al., 2019). The same correlation was obtained on gene expression using qPCR for TRPM8 (Du et al., 2018) or TCGA for TRPM2 (Lin et al., 2018) and TMEM16A (Crottes et al., 2019). Furthermore, the mutation status of TRPM2 was also analyzed using Kaplan-Meier in 10 patients out of 159, and the mutated *TRPM2* gene revealed a negative correlation with patient survival, compared to patients expressing wildtype *TRPM2* (Lin et al., 2018). Studies on shorter cohorts also revealed that high protein expression of Kv11.1 and TRPM7 channels are inversely correlated with overall survival using Pearson correlation on 18 patients, and multivariate overall survival analysis on 44 samples, respectively (Rybarczyk et al., 2012; Lastraioli et al., 2015a) Comparison of ion channels expression level in 9 patients with short survival (<12 months) and 10 patients with long survival (>45 months) showed that short survival was correlated with high expression of CLIC3 and low expression of CLCA1 (Hu et al., 2018b). This low CLCA1 expression correlated with shorter disease-free survival was confirmed using tissue microarrays, immunohistochemistry and Kaplan-Meier analysis in 140 patients (Hu et al., 2018a). Except for CLCA1 which could be proposed as a good prognostic marker, all the other studied ion channels could be proposed as poor prognostic markers.

These studies on human tissues highlighted the major clinical relevance of ion channels expression in pancreatic cancer development (**Table 2** and **Figure 3**). Indeed, the expression of potassium, calcium, chloride channels, and aquaporins is mainly associated with aggressiveness and invasiveness and inversely correlated to patient survival, suggesting that they may be potential markers of poor prognosis.

Therapeutic Targets

In general, PDAC cells are resistant to pro-apoptotic reagents, and overexpression of ion channels was shown to be involved in this resistance. Knockdown of TRPM7 in combination with gemcitabine treatment enhanced cytotoxicity in PANC-1 cells even though the precise mechanisms are not yet determined (Yee et al., 2012b), whereas the silencing of TRPV6 in Capan-2 PDAC cells resulted in a significant increase of sensitivity to the chemotherapeutic reagent oxaliplatin, but had little effect on gemcitabine and cisplatin treatments (Song et al., 2018). Another study has shown that silencing of TRPM8 in combination with gemcitabine suppressed the proliferation and invasion properties of PANC-1 and BxPC-3 cells. In addition, gemcitabine-sensitivity depended on TRPM8 silencing in these cell lines, where mRNA level of multi-drug related proteins was decreased, and expression of apoptosis-related proteins was also affected, suggesting that TRPM8 is involved in multi-drug resistance and apoptosis of PDAC cells (Liu et al., 2018). PANC-1 cells apoptosis was also increased after treatment with chemotherapeutic reagents 5-fluorouracil or gemcitabine in combination with a knockdown of ORAI1, STIM1, or both (Kondratska et al., 2014). Furthermore, it was shown that cells treated with either 5-fluorouracil or gemcitabine increased ORAI1 and STIM1 expression as well as SOC entry suggesting that ORAI1 and STIM1 confer resistance to chemotherapy, probably through the increase of SOC entry (Kondratska et al., 2014). More recently, STIM1 was found to be involved in gemcitabine resistance in PDAC (Zhou et al., 2020). The transcriptome sequencing analysis in established gemcitabine resistant PDAC cell lines, showed that STIM1 was significantly upregulated in the gemcitabine resistant cell lines, compared to the parental cell line (Zhou et al., 2020). Among the chloride channels, knockdown of TMEM16J provided an additive effect on inhibiting proliferation upon treatment with gemcitabine and

erlotinib, suggesting that a TMEM16J inhibitor can help to prevent gemcitabine resistance associated with the prolonged use of gemcitabine (Jun et al., 2017). The team of Arcangeli has been investigating another therapeutic perspective with the development of a novel anti-Kv11.1 antibody-conjugated PEG-TiO_2 nanoparticles for targeting PDAC cells (Sette et al., 2013).

CONCLUSION

Increasing evidence indicates that ion channels are involved in the regulation of cancer proliferation, apoptosis, chemo-resistance, migration, and invasion. The field of ion channels in PDAC still constitutes a novel area of research and even studies conclude their involvement in the malignancy and aggressiveness of PDAC, only relatively few studies provide the complete signaling pathways. Moreover, the majority of the studies cited in this review were carried out on 2D cultured cell lines. It appears thus necessary to develop and/or increase better approaches (organoids, 3D culture, and/or animal models) to investigate the candidate channel(s) as well as its (their) function and associated signaling pathways in PDAC. However, recently, an increasing number of publications on signaling in pancreatic cancer take the tumor microenvironment into-account. This reflects the interest in ionic channels and their potential promising use as therapeutic targets in the fight against pancreatic cancer.

AUTHOR CONTRIBUTIONS

JS, ID-D, AA, and HO-A: design and manuscript preparation. All authors contributed to the article and approved the submitted version.

FUNDING

JS is grateful for the funding by the Marie Skłodowska-Curie Innovative Training Network (ITN) Grant Agreement number: 813834 - pHioniC - H2020-MSCA-ITN-2018. HO-A is grateful for the funding by the Ministère de l'Enseignement Supérieur et de la Recherche, the Région Hauts-de-France (Picardie), the FEDER (Fonds Européen de Développement Économique Régional), the Université Picardie Jules Verne, and the Ligue Contre le Cancer (Septentrion).

REFERENCES

Aichler, M., Seiler, C., Tost, M., Siveke, J., Mazur, P. K., Da Silva-Buttkus, P., et al. (2012). Origin of pancreatic ductal adenocarcinoma from atypical flat lesions: a comparative study in transgenic mice and human tissues. *J. Pathol.* 226 (5), 723–734. doi: 10.1002/path.3017

al-Nakkash, L., and Cotton, C. U. (1997). Bovine pancreatic duct cells express cAMP- and Ca(2+)-activated apical membrane Cl- conductances. *Am. J. Physiol.* 273 (1 Pt 1), G204–G216. doi: 10.1152/ajpgi.1997.273.1.G204

Alvarez-Baron, C. P., Jonsson, P., Thomas, C., Dryer, S. E., and Williams, C. (2011). The two-pore domain potassium channel KCNK5: induction by estrogen receptor alpha and role in proliferation of breast cancer cells. *Mol. Endocrinol.* 25 (8), 1326–1336. doi: 10.1210/me.2011-0045

Anderson, K. J., Cormier, R. T., and Scott, P. M. (2019). Role of ion channels in gastrointestinal cancer. *World J. Gastroenterol.* 25 (38), 5732–5772. doi: 10.3748/wjg.v25.i38.5732

Angus, M., and Ruben, P. (2019). Voltage gated sodium channels in cancer and their potential mechanisms of action. *Channels (Austin)* 13 (1), 400–409. doi: 10.1080/19336950.2019.1666455

Arcangeli, A., Crociani, O., and Bencini, L. (2014). Interaction of tumour cells with their microenvironment: ion channels and cell adhesion molecules. A focus on pancreatic cancer. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 369 (1638), 20130101. doi: 10.1098/rstb.2013.0101

Arsenijevic, T., Perret, J., Van Laethem, J. L., and Delporte, C. (2019). Aquaporins Involvement in Pancreas Physiology and in Pancreatic Diseases. *Int. J. Mol. Sci.* 20 (20). doi: 10.3390/ijms20205052

Ashton, N., Evans, R. L., Elliott, A. C., Green, R., and Argent, B. E. (1993). Regulation of fluid secretion and intracellular messengers in isolated rat pancreatic ducts by acetylcholine. *J. Physiol.* 471, 549–562. doi: 10.1113/jphysiol.1993.sp019915

Ayoub, C., Wasylyk, C., Li, Y., Thomas, E., Marisa, L., Robe, A., et al. (2010). ANO1 amplification and expression in HNSCC with a high propensity for future distant metastasis and its functions in HNSCC cell lines. *Br. J. Cancer* 103 (5), 715–726. doi: 10.1038/sj.bjc.6605823

Bergmann, F., Andrulis, M., Hartwig, W., Penzel, R., Gaida, M. M., Herpel, E., et al. (2011). Discovered on gastrointestinal stromal tumor 1 (DOG1) is expressed in pancreatic centroacinar cells and in solid-pseudopapillary neoplasms–novel evidence for a histogenetic relationship. *Hum. Pathol.* 42 (6), 817–823. doi: 10.1016/j.humpath.2010.10.005

Bielanska, J., Hernandez-Losa, J., Perez-Verdaguer, M., Moline, T., Somoza, R., Ramon, Y. C. S., et al. (2009). Voltage-dependent potassium channels Kv1.3 and Kv1.5 in human cancer. *Curr. Cancer Drug Targets* 9 (8), 904–914. doi: 10.2174/156800909790192400

Bleich, M., and Warth, R. (2000). The very small-conductance K+ channel KvLQT1 and epithelial function. *Pflugers Arch.* 440 (2), 202–206. doi: 10.1007/s004240000257

Bonito, B., Sauter, D. R., Schwab, A., Djamgoz, M. B., and Novak, I. (2016). KCa3.1 (IK) modulates pancreatic cancer cell migration, invasion and proliferation: anomalous effects on TRAM-34. *Pflugers Arch.* 468 (11-12), 1865–1875. doi: 10.1007/s00424-016-1891-9

Brevet, M., Fucks, D., Chatelain, D., Regimbeau, J. M., Delcenserie, R., Sevestre, H., et al. (2009). Deregulation of 2 potassium channels in pancreas adenocarcinomas: implication of KV1.3 gene promoter methylation. *Pancreas* 38 (6), 649–654. doi: 10.1097/MPA.0b013e3181a56ebf

Britschgi, A., Bill, A., Brinkhaus, H., Rothwell, C., Clay, I., Duss, S., et al. (2013). Calcium-activated chloride channel ANO1 promotes breast cancer progression by activating EGFR and CAMK signaling. *Proc. Natl. Acad. Sci. U. S. A.* 110 (11), E1026–E1034. doi: 10.1073/pnas.1217072110

Burghardt, B., Elkaer, M. L., Kwon, T. H., Racz, G. Z., Varga, G., Steward, M. C., et al. (2003). Distribution of aquaporin water channels AQP1 and AQP5 in the ductal system of the human pancreas. *Gut* 52 (7), 1008–1016. doi: 10.1136/gut.52.7.1008

Burghardt, B., Nielsen, S., and Steward, M. C. (2006). The role of aquaporin water channels in fluid secretion by the exocrine pancreas. *J. Membr. Biol.* 210 (2), 143–153. doi: 10.1007/s00232-005-0852-6

Burnstock, G., and Novak, I. (2012). Purinergic signalling in the pancreas in health and disease. *J. Endocrinol.* 213 (2), 123–141. doi: 10.1530/JOE-11-0434

Camacho, J. (2006). Ether a go-go potassium channels and cancer. *Cancer Lett.* 233 (1), 1–9. doi: 10.1016/j.canlet.2005.02.016

Caputo, A., Caci, E., Ferrera, L., Pedemonte, N., Barsanti, C., Sondo, E., et al. (2008). TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. *Science* 322 (5901), 590–594. doi: 10.1126/science.1163518

Case, R. M., and Clausen, T. (1973). The relationship between calcium exchange and enzyme secretion in the isolated rat pancreas. *J. Physiol.* 235 (1), 75–102. doi: 10.1113/jphysiol.1973.sp010379

Castellani, C., and Assael, B. M. (2017). Cystic fibrosis: a clinical view. *Cell Mol. Life Sci.* 74 (1), 129–140. doi: 10.1007/s00018-016-2393-9

Cazacu, I. M., Farkas, N., Garami, A., Balasko, M., Mosdosi, B., Alizadeh, H., et al. (2018). Pancreatitis-Associated Genes and Pancreatic Cancer Risk: A Systematic Review and Meta-analysis. *Pancreas* 47 (9), 1078–1086. doi: 10.1097/MPA.0000000000001145

Chambers, J. A., and Harris, A. (1993). Expression of the cystic fibrosis gene and the major pancreatic mucin gene, MUC1, in human ductal epithelial cells. *J. Cell Sci.* 105 (Pt 2), 417–422.

Chen, C., Vincent, J. D., and Clarke, I. J. (1994). Ion channels and the signal transduction pathways in the regulation of growth hormone secretion. *Trends Endocrinol. Metab.* 5 (6), 227–233. doi: 10.1016/1043-2760(94)p3080-q

Chen, Y. F., Lin, P. C., Yeh, Y. M., Chen, L. H., and Shen, M. R. (2019). Store-Operated Ca(2+) Entry in Tumor Progression: From Molecular Mechanisms to Clinical Implications. *Cancers (Basel)* 11 (7), 899. doi: 10.3390/cancers11070899

Cho, S. J., Sattar, A. K., Jeong, E. H., Satchi, M., Cho, J. A., Dash, S., et al. (2002). Aquaporin 1 regulates GTP-induced rapid gating of water in secretory vesicles. *Proc. Natl. Acad. Sci. U. S. A.* 99 (7), 4720–4724. doi: 10.1073/pnas.072083499

Choi, J. H., Ji, Y. G., Ko, J. J., Cho, H. J., and Lee, D. H. (2018). Activating P2X7 Receptors Increases Proliferation of Human Pancreatic Cancer Cells via ERK1/2 and JNK. *Pancreas* 47 (5), 643–651. doi: 10.1097/MPA.0000000000001055

Chow, J. Y., Dong, H., Quach, K. T., Van Nguyen, P. N., Chen, K., and Carethers, J. M. (2008). TGF-beta mediates PTEN suppression and cell motility through calcium-dependent PKC-alpha activation in pancreatic cancer cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* 294 (4), G899–G905. doi: 10.1152/ajpgi.00411.2007

Comes, N., Bielanska, J., Vallejo-Gracia, A., Serrano-Albarras, A., Marruecos, L., Gomez, D., et al. (2013). The voltage-dependent K(+) channels Kv1.3 and Kv1.5 in human cancer. *Front. Physiol.* 4, 283. doi: 10.3389/fphys.2013.00283

Comes, N., Serrano-Albarras, A., Capera, J., Serrano-Novillo, C., Condom, E., Ramon, Y. C. S., et al. (2015). Involvement of potassium channels in the progression of cancer to a more malignant phenotype. *Biochim. Biophys. Acta* 1848 (10 Pt B), 2477–2492. doi: 10.1016/j.bbamem.2014.12.008

Coradini, D., Casarsa, C., and Oriana, S. (2011). Epithelial cell polarity and tumorigenesis: new perspectives for cancer detection and treatment. *Acta Pharmacol. Sin.* 32 (5), 552–564. doi: 10.1038/aps.2011.20

Crottes, D., Lin, Y. T., Peters, C. J., Gilchrist, J. M., Wiita, A. P., Jan, Y. N., et al. (2019). TMEM16A controls EGF-induced calcium signaling implicated in pancreatic cancer prognosis. *Proc. Natl. Acad. Sci. U. S. A.* 116 (26), 13026–13035. doi: 10.1073/pnas.1900703116

Cucu, D., Chiritoiu, G., Petrescu, S., Babes, A., Stanica, L., Duda, D. G., et al. (2014). Characterization of functional transient receptor potential melastatin 8 channels in human pancreatic ductal adenocarcinoma cells. *Pancreas* 43 (5), 795–800. doi: 10.1097/MPA.0000000000000106

Delporte, C. (2014). Aquaporins in salivary glands and pancreas. *Biochim. Biophys. Acta* 1840 (5), 1524–1532. doi: 10.1016/j.bbagen.2013.08.007

Demolombe, S., Franco, D., de Boer, P., Kuperschmidt, S., Roden, D., Pereon, Y., et al. (2001). Differential expression of KvLQT1 and its regulator IsK in mouse epithelia. *Am. J. Physiol. Cell Physiol.* 280 (2), C359–C372. doi: 10.1152/ajpcell.2001.280.2.C359

Direito, I., Paulino, J., Vigia, E., Brito, M. A., and Soveral, G. (2017). Differential expression of aquaporin-3 and aquaporin-5 in pancreatic ductal adenocarcinoma. *J. Surg. Oncol.* 115 (8), 980–996. doi: 10.1002/jso.24605

Djamgoz, M. B., Coombes, R. C., and Schwab, A. (2014). Ion transport and cancer: from initiation to metastasis. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 369 (1638), 20130092. doi: 10.1098/rstb.2013.0092

Domotor, A., Peidl, Z., Vincze, A., Hunyady, B., Szolcsanyi, J., Kereskay, L., et al. (2005). Immunohistochemical distribution of vanilloid receptor, calcitonin-gene related peptide and substance P in gastrointestinal mucosa of patients with different gastrointestinal disorders. *Inflammopharmacology* 13 (1-3), 161–177. doi: 10.1163/156856005774423737

Dong, H., Shim, K. N., Li, J. M., Estrema, C., Ornelas, T. A., Nguyen, F., et al. (2010). Molecular mechanisms underlying Ca2+-mediated motility of human pancreatic duct cells. *Am. J. Physiol. Cell Physiol.* 299 (6), C1493–C1503. doi: 10.1152/ajpcell.00242.2010

Dozynkiewicz, M. A., Jamieson, N. B., Macpherson, I., Grindlay, J., van den Berghe, P. V., von Thun, A., et al. (2012). Rab25 and CLIC3 collaborate to promote integrin recycling from late endosomes/lysosomes and drive cancer progression. *Dev. Cell* 22 (1), 131–145. doi: 10.1016/j.devcel.2011.11.008

Du, J. D., Zheng, X., Chen, Y. L., Huang, Z. Q., Cai, S. W., Jiao, H. B., et al. (2018). Elevated Transient Receptor Potential Melastatin 8 (TRPM8) Expression Is Correlated with Poor Prognosis in Pancreatic Cancer. *Med. Sci. Monit.* 24, 3720–3725. doi: 10.12659/MSM.909968

Duprat, F., Lesage, F., Fink, M., Reyes, R., Heurteaux, C., and Lazdunski, M. (1997). TASK, a human background K+ channel to sense external pH variations near physiological pH. *EMBO J.* 16 (17), 5464–5471. doi: 10.1093/emboj/16.17.5464

Duprat, F., Girard, C., Jarretou, G., and Lazdunski, M. (2005). Pancreatic two P domain K+ channels TALK-1 and TALK-2 are activated by nitric oxide and reactive oxygen species. *J. Physiol.* 562 (Pt 1), 235–244. doi: 10.1113/jphysiol.2004.071266

Duran, C., Thompson, C. H., Xiao, Q., and Hartzell, H. C. (2010). Chloride channels: often enigmatic, rarely predictable. *Annu. Rev. Physiol.* 72, 95–121. doi: 10.1146/annurev-physiol-021909-135811

Evans, R. L., Ashton, N., Elliott, A. C., Green, R., and Argent, B. E. (1996). Interactions between secretin and acetylcholine in the regulation of fluid secretion by isolated rat pancreatic ducts. *J. Physiol.* 496 (Pt 1), 265–273. doi: 10.1113/jphysiol.1996.sp021683

Falzone, M. E., Malvezzi, M., Lee, B. C., and Accardi, A. (2018). Known structures and unknown mechanisms of TMEM16 scramblases and channels. *J. Gen. Physiol.* 150 (7), 933–947. doi: 10.1085/jgp.201711957

Feng, J., Yu, J., Pan, X., Li, Z., Chen, Z., Zhang, W., et al. (2014). HERG1 functions as an oncogene in pancreatic cancer and is downregulated by miR-96. *Oncotarget* 5 (14), 5832–5844. doi: 10.18632/oncotarget.2200

Fong, P., Argent, B. E., Guggino, W. B., and Gray, M. A. (2003). Characterization of vectorial chloride transport pathways in the human pancreatic duct adenocarcinoma cell line HPAF. *Am. J. Physiol. Cell Physiol.* 285 (2), C433–C445. doi: 10.1152/ajpcell.00509.2002

Furuya, S., Naruse, S., Ko, S. B., Ishiguro, H., Yoshikawa, T., and Hayakawa, T. (2002). Distribution of aquaporin 1 in the rat pancreatic duct system examined with light- and electron-microscopic immunohistochemistry. *Cell Tissue Res.* 308 (1), 75–86. doi: 10.1007/s00441-002-0527-x

Gabbi, C., Kim, H. J., Hultenby, K., Bouton, D., Toresson, G., Warner, M., et al. (2008). Pancreatic exocrine insufficiency in LXRbeta-/- mice is associated with a reduction in aquaporin-1 expression. *Proc. Natl. Acad. Sci. U. S. A.* 105 (39), 15052–15057. doi: 10.1073/pnas.0808097105

Gallacher, D. V., Maruyama, Y., and Petersen, O. H. (1984). Patch-clamp study of rubidium and potassium conductances in single cation channels from mammalian exocrine acini. *Pflugers Arch.* 401 (4), 361–367. doi: 10.1007/BF00584336

Gautam, D., Han, S. J., Heard, T. S., Cui, Y., Miller, G., Bloodworth, L., et al. (2005). Cholinergic stimulation of amylase secretion from pancreatic acinar cells studied with muscarinic acetylcholine receptor mutant mice. *J. Pharmacol. Exp. Ther.* 313 (3), 995–1002. doi: 10.1124/jpet.105.084855

Gerasimenko, J. V., Gryshchenko, O., Ferdek, P. E., Stapleton, E., Hebert, T. O., Bychkova, S., et al. (2013). Ca2+ release-activated Ca2+ channel blockade as a potential tool in antipancreatitis therapy. *Proc. Natl. Acad. Sci. U. S. A.* 110 (32), 13186–13191. doi: 10.1073/pnas.1300910110

Gheldof, A., and Berx, G. (2013). Cadherins and epithelial-to-mesenchymal transition. *Prog. Mol. Biol. Transl. Sci.* 116, 317–336. doi: 10.1016/B978-0-12-394311-8.00014-5

Giannuzzo, A., Pedersen, S. F., and Novak, I. (2015). The P2X7 receptor regulates cell survival, migration and invasion of pancreatic ductal adenocarcinoma cells. *Mol. Cancer* 14, 203. doi: 10.1186/s12943-015-0472-4

Giannuzzo, A., Saccomano, M., Napp, J., Ellegaard, M., Alves, F., and Novak, I. (2016). Targeting of the P2X7 receptor in pancreatic cancer and stellate cells. *Int. J. Cancer* 139 (11), 2540–2552. doi: 10.1002/ijc.30380

Giovannucci, D. R., Bruce, J. I., Straub, S. V., Arreola, J., Sneyd, J., Shuttleworth, T. J., et al. (2002). Cytosolic Ca(2+) and Ca(2+)-activated Cl(-) current dynamics: insights from two functionally distinct mouse exocrine cells. *J. Physiol.* 540 (Pt 2), 469–484. doi: 10.1113/jphysiol.2001.013453

Girard, C., Duprat, F., Terrenoire, C., Tinel, N., Fosset, M., Romey, G., et al. (2001). Genomic and functional characteristics of novel human pancreatic 2P domain K(+) channels. *Biochem. Biophys. Res. Commun.* 282 (1), 249–256. doi: 10.1006/bbrc.2001.4562

Gomez-Varela, D., Zwick-Wallasch, E., Knotgen, H., Sanchez, A., Hettmann, T., Ossipov, D., et al. (2007). Monoclonal antibody blockade of the human Eag1 potassium channel function exerts antitumor activity. *Cancer Res.* 67 (15), 7343–7349. doi: 10.1158/0008-5472.CAN-07-0107

Gouaux, E., and Mackinnon, R. (2005). Principles of selective ion transport in channels and pumps. *Science* 310 (5753), 1461–1465. doi: 10.1126/science.1113666

Gray, M. A., Greenwell, J. R., and Argent, B. E. (1988). Secretin-regulated chloride channel on the apical plasma membrane of pancreatic duct cells. *J. Membr. Biol.* 105 (2), 131–142. doi: 10.1007/BF02009166

Gray, M. A., Harris, A., Coleman, L., Greenwell, J. R., and Argent, B. E. (1989). Two types of chloride channel on duct cells cultured from human fetal pancreas. *Am. J. Physiol.* 257 (2 Pt 1), C240–C251. doi: 10.1152/ajpcell.1989.257.2.C240

Gray, M. A., Greenwell, J. R., Garton, A. J., and Argent, B. E. (1990a). Regulation of maxi-K+ channels on pancreatic duct cells by cyclic AMP-dependent phosphorylation. *J. Membr. Biol.* 115 (3), 203–215. doi: 10.1007/bf01868636

Gray, M. A., Pollard, C. E., Harris, A., Coleman, L., Greenwell, J. R., and Argent, B. E. (1990b). Anion selectivity and block of the small-conductance chloride channel on pancreatic duct cells. *Am. J. Physiol.* 259 (5 Pt 1), C752–C761. doi: 10.1152/ajpcell.1990.259.5.C752

Gray, M. A., Plant, S., and Argent, B. E. (1993). cAMP-regulated whole cell chloride currents in pancreatic duct cells. *Am. J. Physiol.* 264 (3 Pt 1), C591–C602. doi: 10.1152/ajpcell.1993.264.3.C591

Gray, M. A., Winpenny, J. P., Porteous, D. J., Dorin, J. R., and Argent, B. E. (1994). CFTR and calcium-activated chloride currents in pancreatic duct cells of a transgenic CF mouse. *Am. J. Physiol.* 266 (1 Pt 1), C213–C221. doi: 10.1152/ajpcell.1994.266.1.C213

Gray, M. A., Winpenny, J. P., Verdon, B., McAlroy, H., and Argent, B. E. (1995). Chloride channels and cystic fibrosis of the pancreas. *Biosci. Rep.* 15 (6), 531–541. doi: 10.1007/BF01204355

Grimmig, T., Matthes, N., Hoeland, K., Tripathi, S., Chandraker, A., Grimm, M., et al. (2015). TLR7 and TLR8 expression increases tumor cell proliferation and promotes chemoresistance in human pancreatic cancer. *Int. J. Oncol.* 47 (3), 857–866. doi: 10.3892/ijo.2015.3069

Hamoir, C., Pepermans, X., Piessevaux, H., Jouret-Mourin, A., Weynand, B., Habyalimana, J. B., et al. (2013). Clinical and morphological characteristics of sporadic genetically determined pancreatitis as compared to idiopathic pancreatitis: higher risk of pancreatic cancer in CFTR variants. *Digestion* 87 (4), 229–239. doi: 10.1159/000348439

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* 144 (5), 646–674. doi: 10.1016/j.cell.2011.02.013

Hansen, M. R., Krabbe, S., and Novak, I. (2008). Purinergic receptors and calcium signalling in human pancreatic duct cell lines. *Cell Physiol. Biochem.* 22 (1-4), 157–168. doi: 10.1159/000149793

Hartel, M., di Mola, F. F., Selvaggi, F., Mascetta, G., Wente, M. N., Felix, K., et al. (2006). Vanilloids in pancreatic cancer: potential for chemotherapy and pain management. *Gut* 55 (4), 519–528. doi: 10.1136/gut.2005.073205

Hayashi, M., and Novak, I. (2013). Molecular basis of potassium channels in pancreatic duct epithelial cells. *Channels (Austin)* 7 (6), 432–441. doi: 10.4161/chan.26100

Hayashi, M., Wang, J., Hede, S. E., and Novak, I. (2012). An intermediate-conductance Ca2+-activated K+ channel is important for secretion in pancreatic duct cells. *Am. J. Physiol. Cell Physiol.* 303 (2), C151–C159. doi: 10.1152/ajpcell.00089.2012

Hede, S. E., Amstrup, J., Christoffersen, B. C., and Novak, I. (1999). Purinoceptors evoke different electrophysiological responses in pancreatic ducts. P2Y inhibits K(+) conductance, and P2X stimulates cation conductance. *J. Biol. Chem.* 274 (45), 31784–31791. doi: 10.1074/jbc.274.45.31784

Hede, S. E., Amstrup, J., Klaerke, D. A., and Novak, I. (2005). P2Y2 and P2Y4 receptors regulate pancreatic Ca(2+)-activated K+ channels differently. *Pflugers Arch.* 450 (6), 429–436. doi: 10.1007/s00424-005-1433-3

Hegyi, P., and Petersen, O. H. (2013). The exocrine pancreas: the acinar-ductal tango in physiology and pathophysiology. *Rev. Physiol. Biochem. Pharmacol.* 165, 1–30. doi: 10.1007/112_2013_14

Hemmerlein, B., Weseloh, R. M., Mello de Queiroz, F., Knotgen, H., Sanchez, A., Rubio, M. E., et al. (2006). Overexpression of Eag1 potassium channels in clinical tumours. *Mol. Cancer* 5:41. doi: 10.1186/1476-4598-5-41

Hennig, A., Wolf, L., Jahnke, B., Polster, H., Seidlitz, T., Werner, K., et al. (2019). CFTR Expression Analysis for Subtyping of Human Pancreatic Cancer Organoids. *Stem Cells Int.* 2019:1024614. doi: 10.1155/2019/1024614

Hong, J. H., Li, Q., Kim, M. S., Shin, D. M., Feske, S., Birnbaumer, L., et al. (2011). Polarized but differential localization and recruitment of STIM1, Orai1 and TRPC channels in secretory cells. *Traffic* 12 (2), 232–245. doi: 10.1111/j.1600-0854.2010.01138.x

Hoth, M., and Niemeyer, B. A. (2013). The neglected CRAC proteins: Orai2, Orai3, and STIM2. *Curr. Top. Membr.* 71, 237–271. doi: 10.1016/B978-0-12-407870-3.00010-X

Hu, D., Ansari, D., Pawlowski, K., Zhou, Q., Sasor, A., Welinder, C., et al. (2018a). Proteomic analyses identify prognostic biomarkers for pancreatic ductal adenocarcinoma. *Oncotarget* 9 (11), 9789–9807. doi: 10.18632/oncotarget.23929

Hu, D., Ansari, D., Zhou, Q., Sasor, A., Hilmersson, K. S., Bauden, M., et al. (2018b). Calcium-activated chloride channel regulator 1 as a prognostic biomarker in pancreatic ductal adenocarcinoma. *BMC Cancer* 18 (1), 1096. doi: 10.1186/s12885-018-5013-2

Hu, D., Ansari, D., Bauden, M., Zhou, Q., and Andersson, R. (2019). The Emerging Role of Calcium-activated Chloride Channel Regulator 1 in Cancer. *Anticancer Res.* 39 (4), 1661–1666. doi: 10.21873/anticanres.13271

Huang, F., Rock, J. R., Harfe, B. D., Cheng, T., Huang, X., Jan, Y. N., et al. (2009). Studies on expression and function of the TMEM16A calcium-activated chloride channel. *Proc. Natl. Acad. Sci. U. S. A.* 106 (50), 21413–21418. doi: 10.1073/pnas.0911935106

Huang, X., Huang, L., and Shao, M. (2017). Aquaporin 3 facilitates tumor growth in pancreatic cancer by modulating mTOR signaling. *Biochem. Biophys. Res. Commun.* 486 (4), 1097–1102. doi: 10.1016/j.bbrc.2017.03.168

Huang, J., Liu, J., and Qiu, L. (2020). Transient receptor potential vanilloid 1 promotes EGFR ubiquitination and modulates EGFR/MAPK signalling in pancreatic cancer cells. *Cell Biochem. Funct.* 38 (4), 401–408. doi: 10.1002/cbf.3483

Hurley, P. T., Ferguson, C. J., Kwon, T. H., Andersen, M. L., Norman, A. G., Steward, M. C., et al. (2001). Expression and immunolocalization of aquaporin water channels in rat exocrine pancreas. *Am. J. Physiol. Gastrointest. Liver Physiol.* 280 (4), G701–G709. doi: 10.1152/ajpgi.2001.280.4.G701

Ishiguro, H., Naruse, S., Kitagawa, M., Mabuchi, T., Kondo, T., Hayakawa, T., et al. (2002). Chloride transport in microperfused interlobular ducts isolated from guinea-pig pancreas. *J. Physiol.* 539 (Pt 1), 175–189. doi: 10.1113/jphysiol.2001.012490

Ishiguro, H., Steward, M. C., Naruse, S., Ko, S. B., Goto, H., Case, R. M., et al. (2009). CFTR functions as a bicarbonate channel in pancreatic duct cells. *J. Gen. Physiol.* 133 (3), 315–326. doi: 10.1085/jgp.200810122

Ishiguro, H., Yamamoto, A., Nakakuki, M., Yi, L., Ishiguro, M., Yamaguchi, M., et al. (2012). Physiology and pathophysiology of bicarbonate secretion by pancreatic duct epithelium. *Nagoya J. Med. Sci.* 74 (1-2), 1–18.

Ishii, T. M., Silvia, C., Hirschberg, B., Bond, C. T., Adelman, J. P., and Maylie, J. (1997). A human intermediate conductance calcium-activated potassium channel. *Proc. Natl. Acad. Sci. U. S. A.* 94 (21), 11651–11656. doi: 10.1073/pnas.94.21.11651

Isokpehi, R. D., Rajnarayanan, R. V., Jeffries, C. D., Oyeleye, T. O., and Cohly, H. H. (2009). Integrative sequence and tissue expression profiling of chicken and mammalian aquaporins. *BMC Genomics* 10 Suppl 2, S7. doi: 10.1186/1471-2164-10-S2-S7

Itoh, T., Rai, T., Kuwahara, M., Ko, S. B., Uchida, S., Sasaki, S., et al. (2005). Identification of a novel aquaporin, AQP12, expressed in pancreatic acinar cells. *Biochem. Biophys. Res. Commun.* 330 (3), 832–838. doi: 10.1016/j.bbrc.2005.03.046

Iwatsuki, N., and Petersen, O. H. (1977). Pancreatic acinar cells: localization of acetylcholine receptors and the importance of chloride and calcium for acetylcholine-evoked depolarization. *J. Physiol.* 269 (3), 723–733. doi: 10.1113/jphysiol.1977.sp011925

Iwatsuki, N., and Petersen, O. H. (1985a). Action of tetraethylammonium on calcium-activated potassium channels in pig pancreatic acinar cells studied by patch-clamp single-channel and whole-cell current recording. *J. Membr. Biol.* 86 (2), 139–144. doi: 10.1007/BF01870780

Iwatsuki, N., and Petersen, O. H. (1985b). Inhibition of Ca2+-activated K+ channels in pig pancreatic acinar cells by Ba2+, Ca2+, quinine and quinidine. *Biochim. Biophys. Acta* 819 (2), 249–257. doi: 10.1016/0005-2736(85)90180-4

Jabbar, K. S., Arike, L., Verbeke, C. S., Sadik, R., and Hansson, G. C. (2018). Highly Accurate Identification of Cystic Precursor Lesions of Pancreatic Cancer Through Targeted Mass Spectrometry: A Phase IIc Diagnostic Study. *J. Clin. Oncol.* 36 (4), 367–375. doi: 10.1200/JCO.2017.73.7288

Jager, H., Dreker, T., Buck, A., Giehl, K., Gress, T., and Grissmer, S. (2004). Blockage of intermediate-conductance Ca2+-activated K+ channels inhibit human pancreatic cancer cell growth in vitro. *Mol. Pharmacol.* 65 (3), 630–638. doi: 10.1124/mol.65.3.630

Jia, N., Dong, S., Zhao, G., Gao, H., Li, X., and Zhang, H. (2016). CLIC1 overexpression is associated with poor prognosis in pancreatic ductal adenocarcinomas. *J. Cancer Res. Ther.* 12 (2), 892–896. doi: 10.4103/0973-1482.154057

Jiang, S., Zhu, L., Yang, J., Hu, L., Gu, J., Xing, X., et al. (2017). Integrated expression profiling of potassium channels identifys KCNN4 as a prognostic biomarker of pancreatic cancer. *Biochem. Biophys. Res. Commun.* 494 (1-2), 113–119. doi: 10.1016/j.bbrc.2017.10.072

Joiner, W. J., Wang, L. Y., Tang, M. D., and Kaczmarek, L. K. (1997). hSK4, a member of a novel subfamily of calcium-activated potassium channels. *Proc. Natl. Acad. Sci. U. S. A.* 94 (20), 11013–11018. doi: 10.1073/pnas.94.20.11013

Jun, I., Park, H. S., Piao, H., Han, J. W., An, M. J., Yun, B. G., et al. (2017). ANO9/TMEM16J promotes tumourigenesis via EGFR and is a novel therapeutic target for pancreatic cancer. *Br. J. Cancer* 117 (12), 1798–1809. doi: 10.1038/bjc.2017.355

Jung, S. R., Kim, K., Hille, B., Nguyen, T. D., and Koh, D. S. (2006). Pattern of Ca2+ increase determines the type of secretory mechanism activated in dog pancreatic duct epithelial cells. *J. Physiol.* 576 (Pt 1), 163–178. doi: 10.1113/jphysiol.2006.114876

Kalman, K., Nguyen, A., Tseng-Crank, J., Dukes, I. D., Chandy, G., Hustad, C. M., et al. (1998). Genomic organization, chromosomal localization, tissue distribution, and biophysical characterization of a novel mammalian Shaker-related voltage-gated potassium channel, Kv1.7. *J. Biol. Chem.* 273 (10), 5851–5857. doi: 10.1074/jbc.273.10.5851

Kasai, H., and Augustine, G. J. (1990). Cytosolic Ca2+ gradients triggering unidirectional fluid secretion from exocrine pancreas. *Nature* 348 (6303), 735–738. doi: 10.1038/348735a0

Khan, H. Y., Mpilla, G. B., Sexton, R., Viswanadha, S., Penmetsa, K. V., Aboukameel, A., et al. (2020). Calcium Release-Activated Calcium (CRAC) Channel Inhibition Suppresses Pancreatic Ductal Adenocarcinoma Cell Proliferation and Patient-Derived Tumor Growth. *Cancers (Basel)* 12 (3), 750. doi: 10.3390/cancers12030750

Kim, S. J., and Greger, R. (1999). Voltage-dependent, slowly activating K+ current (I(Ks)) and its augmentation by carbachol in rat pancreatic acini. *Pflugers Arch.* 438 (5), 604–611. doi: 10.1007/s004249900071

Kim, S. J., Kerst, G., Schreiber, R., Pavenstadt, H., Greger, R., Hug, M. J., et al. (2000). Inwardly rectifying K+ channels in the basolateral membrane of rat pancreatic acini. *Pflugers Arch.* 441 (2-3), 331–340. doi: 10.1007/s004240000427

Kim, C. J., Cho, Y. G., Jeong, S. W., Kim, Y. S., Kim, S. Y., Nam, S. W., et al. (2004). Altered expression of KCNK9 in colorectal cancers. *APMIS* 112 (9), 588–594. doi: 10.1111/j.1600-0463.2004.apm1120905.x

Kim, J. Y., Zeng, W., Kiselyov, K., Yuan, J. P., Dehoff, M. H., Mikoshiba, K., et al. (2006). Homer 1 mediates store- and inositol 1,4,5-trisphosphate receptor-dependent translocation and retrieval of TRPC3 to the plasma membrane. *J. Biol. Chem.* 281 (43), 32540–32549. doi: 10.1074/jbc.M602496200

Kim, M. H., Choi, B. H., Jung, S. R., Sernka, T. J., Kim, S., Kim, K. T., et al. (2008). Protease-activated receptor-2 increases exocytosis via multiple signal transduction pathways in pancreatic duct epithelial cells. *J. Biol. Chem.* 283 (27), 18711–18720. doi: 10.1074/jbc.M801655200

Kim, M. S., Hong, J. H., Li, Q., Shin, D. M., Abramowitz, J., Birnbaumer, L., et al. (2009). Deletion of TRPC3 in mice reduces store-operated Ca2+ influx and the severity of acute pancreatitis. *Gastroenterology* 137 (4), 1509–1517. doi: 10.1053/j.gastro.2009.07.042

Kim, M. H., Seo, J. B., Burnett, L. A., Hille, B., and Koh, D. S. (2013). Characterization of store-operated Ca2+ channels in pancreatic duct epithelia. *Cell Calcium* 54 (4), 266–275. doi: 10.1016/j.ceca.2013.07.002

Kim, H., Kim, H., Lee, J., Lee, B., Kim, H. R., Jung, J., et al. (2018). Anoctamin 9/TMEM16J is a cation channel activated by cAMP/PKA signal. *Cell Calcium* 71, 75–85. doi: 10.1016/j.ceca.2017.12.003

Kim, J. B. (2014). Channelopathies. *Korean J. Pediatr.* 57 (1), 1–18. doi: 10.3345/kjp.2014.57.1.1

Kirkegard, J., Mortensen, F. V., and Cronin-Fenton, D. (2017). Chronic Pancreatitis and Pancreatic Cancer Risk: A Systematic Review and Meta-analysis. *Am. J. Gastroenterol.* 112 (9), 1366–1372. doi: 10.1038/ajg.2017.218

Ko, S. B., Naruse, S., Kitagawa, M., Ishiguro, H., Furuya, S., Mizuno, N., et al. (2002). Aquaporins in rat pancreatic interlobular ducts. *Am. J. Physiol. Gastrointest. Liver Physiol.* 282 (2), G324–G331. doi: 10.1152/ajpgi.00198.2001

Koltai, T. (2015). Voltage-gated sodium channel as a target for metastatic risk reduction with re-purposed drugs. *F1000Res* 4, 297. doi: 10.12688/f1000research.6789.1

Kondratska, K., Kondratskyi, A., Yassine, M., Lemonnier, L., Lepage, G., Morabito, A., et al. (2014). Orai1 and STIM1 mediate SOCE and contribute to apoptotic resistance of pancreatic adenocarcinoma. *Biochim. Biophys. Acta* 1843 (10), 2263–2269. doi: 10.1016/j.bbamcr.2014.02.012

Kong, S. C., Giannuzzo, A., Novak, I., and Pedersen, S. F. (2014). Acid-base transport in pancreatic cancer: molecular mechanisms and clinical potential. *Biochem. Cell Biol.* 92 (6), 449–459. doi: 10.1139/bcb-2014-0078

Kottgen, M., Hoefer, A., Kim, S. J., Beschorner, U., Schreiber, R., Hug, M. J., et al. (1999). Carbachol activates a K+ channel of very small conductance in the basolateral membrane of rat pancreatic acinar cells. *Pflugers Arch.* 438 (5), 597–603. doi: 10.1007/s004249900070

Kovalenko, I., Glasauer, A., Schockel, L., Sauter, D. R., Ehrmann, A., Sohler, F., et al. (2016). Identification of KCa3.1 Channel as a Novel Regulator of Oxidative Phosphorylation in a Subset of Pancreatic Carcinoma Cell Lines. *PloS One* 11 (8), e0160658. doi: 10.1371/journal.pone.0160658

Koyama, Y., Yamamoto, T., Kondo, D., Funaki, H., Yaoita, E., Kawasaki, K., et al. (1997). Molecular cloning of a new aquaporin from rat pancreas and liver. *J. Biol. Chem.* 272 (48), 30329–30333. doi: 10.1074/jbc.272.48.30329

Kunzelmann, K. (2005). Ion channels and cancer. *J. Membr. Biol.* 205 (3), 159–173. doi: 10.1007/s00232-005-0781-4

Kunzelmann, K. (2016). Ion channels in regulated cell death. *Cell Mol. Life Sci.* 73 (11-12), 2387–2403. doi: 10.1007/s00018-016-2208-z

Kunzli, B. M., Berberat, P. O., Giese, T., Csizmadia, E., Kaczmarek, E., Baker, C., et al. (2007). Upregulation of CD39/NTPDases and P2 receptors in human pancreatic disease. *Am. J. Physiol. Gastrointest. Liver Physiol.* 292 (1), G223–G230. doi: 10.1152/ajpgi.00259.2006

Lang, F., and Stournaras, C. (2014). Ion channels in cancer: future perspectives and clinical potential. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 369 (1638), 20130108. doi: 10.1098/rstb.2013.0108

Lastraioli, E., Lottini, T., Bencini, L., Bernini, M., and Arcangeli, A. (2015a). hERG1 Potassium Channels: Novel Biomarkers in Human Solid Cancers. *BioMed. Res. Int.* 2015:896432. doi: 10.1155/2015/896432

Lastraioli, E., Perrone, G., Sette, A., Fiore, A., Crociani, O., Manoli, S., et al. (2015b). hERG1 channels drive tumour malignancy and may serve as prognostic factor in pancreatic ductal adenocarcinoma. *Br. J. Cancer* 112 (6), 1076–1087. doi: 10.1038/bjc.2015.28

Lazzeri, M., Vannucchi, M. G., Spinelli, M., Bizzoco, E., Beneforti, P., Turini, D., et al. (2005). Transient receptor potential vanilloid type 1 (TRPV1) expression changes from normal urothelium to transitional cell carcinoma of human bladder. *Eur. Urol.* 48 (4), 691–698. doi: 10.1016/j.eururo.2005.05.018

Lee, M. G., Ahn, W., Lee, J. A., Kim, J. Y., Choi, J. Y., Moe, O. W., et al. (2001). Coordination of pancreatic HCO3- secretion by protein-protein interaction between membrane transporters. *JOP* 2 (4 Suppl), 203–206.

Lee, G. W., Park, H. S., Kim, E. J., Cho, Y. W., Kim, G. T., Mun, Y. J., et al. (2012). Reduction of breast cancer cell migration via up-regulation of TASK-3 two-pore domain K+ channel. *Acta Physiol. (Oxf)* 204 (4), 513–524. doi: 10.1111/j.1748-1716.2011.02359.x

Lee, M. G., Ohana, E., Park, H. W., Yang, D., and Muallem, S. (2012). Molecular mechanism of pancreatic and salivary gland fluid and HCO3 secretion. *Physiol. Rev.* 92 (1), 39–74. doi: 10.1152/physrev.00011.2011

Lemstrova, R., Soucek, P., Melichar, B., and Mohelnikova-Duchonova, B. (2014). Role of solute carrier transporters in pancreatic cancer: a review. *Pharmacogenomics* 15 (8), 1133–1145. doi: 10.2217/pgs.14.80

Li, L., and Hanahan, D. (2013). Hijacking the neuronal NMDAR signaling circuit to promote tumor growth and invasion. *Cell* 153 (1), 86–100. doi: 10.1016/j.cell.2013.02.051

Liddle, R. A. (2007). The role of Transient Receptor Potential Vanilloid 1 (TRPV1) channels in pancreatitis. *Biochim. Biophys. Acta* 1772 (8), 869–878. doi: 10.1016/j.bbadis.2007.02.012

Lin, R., Wang, Y., Chen, Q., Liu, Z., Xiao, S., Wang, B., et al. (2018). TRPM2 promotes the proliferation and invasion of pancreatic ductal adenocarcinoma. *Mol. Med. Rep.* 17 (6), 7537–7544. doi: 10.3892/mmr.2018.8816

Litan, A., and Langhans, S. A. (2015). Cancer as a channelopathy: ion channels and pumps in tumor development and progression. *Front. Cell Neurosci.* 9, 86. doi: 10.3389/fncel.2015.00086

Liu, Y., McKenna, E., Figueroa, D. J., Blevins, R., Austin, C. P., Bennett, P. B., et al. (2000). The human inward rectifier K(+) channel subunit kir5.1 (KCNJ16) maps to chromosome 17q25 and is expressed in kidney and pancreas. *Cytogenet. Cell Genet.* 90 (1-2), 60–63. doi: 10.1159/000015662

Liu, X., Cheng, K. T., Bandyopadhyay, B. C., Pani, B., Dietrich, A., Paria, B. C., et al. (2007). Attenuation of store-operated Ca2+ current impairs salivary gland fluid secretion in TRPC1(-/-) mice. *Proc. Natl. Acad. Sci. U. S. A.* 104 (44), 17542–17547. doi: 10.1073/pnas.0701254104

Liu, W., Lu, M., Liu, B., Huang, Y., and Wang, K. (2012). Inhibition of Ca(2+)-activated Cl(-) channel ANO1/TMEM16A expression suppresses tumor growth and invasiveness in human prostate carcinoma. *Cancer Lett.* 326 (1), 41–51. doi: 10.1016/j.canlet.2012.07.015

Liu, J., Hu, G., Gong, Y., Yu, Q., He, B., Li, W., et al. (2018). Silencing of TRPM8 inhibits aggressive tumor phenotypes and enhances gemcitabine sensitivity in pancreatic cancer. *Pancreatology* 18 (8), 935–944. doi: 10.1016/j.pan.2018.08.011

Lotz, M. M., Wang, H., Song, J. C., Pories, S. E., and Matthews, J. B. (2004). K+ channel inhibition accelerates intestinal epithelial cell wound healing. *Wound Repair Regener.* 12 (5), 565–574. doi: 10.1111/j.1067-1927.2004.012509.x

Lowenfels, A. B., Maisonneuve, P., Cavallini, G., Ammann, R. W., Lankisch, P. G., Andersen, J. R., et al. (1993). Pancreatitis and the risk of pancreatic cancer. International Pancreatitis Study Group. *N. Engl. J. Med.* 328 (20), 1433–1437. doi: 10.1056/NEJM199305203282001

Lu, J., Dong, Q., Zhang, B., Wang, X., Ye, B., Zhang, F., et al. (2015). Chloride intracellular channel 1 (CLIC1) is activated and functions as an oncogene in pancreatic cancer. *Med. Oncol.* 32 (6), 616. doi: 10.1007/s12032-015-0616-9

Lur, G., Haynes, L. P., Prior, I. A., Gerasimenko, O. V., Feske, S., Petersen, O. H., et al. (2009). Ribosome-free terminals of rough ER allow formation of STIM1 puncta and segregation of STIM1 from IP(3) receptors. *Curr. Biol.* 19 (19), 1648–1653. doi: 10.1016/j.cub.2009.07.072

Lur, G., Sherwood, M. W., Ebisui, E., Haynes, L., Feske, S., Sutton, R., et al. (2011). InsP(3)receptors and Orai channels in pancreatic acinar cells: co-localization and its consequences. *Biochem. J.* 436 (2), 231–239. doi: 10.1042/BJ20110083

Ma, T., Jayaraman, S., Wang, K. S., Song, Y., Yang, B., Li, J., et al. (2001). Defective dietary fat processing in transgenic mice lacking aquaporin-1 water channels. *Am. J. Physiol. Cell Physiol.* 280 (1), C126–C134. doi: 10.1152/ajpcell.2001.280.1.C126

Maleth, J., and Hegyi, P. (2014). Calcium signaling in pancreatic ductal epithelial cells: an old friend and a nasty enemy. *Cell Calcium* 55 (6), 337–345. doi: 10.1016/j.ceca.2014.02.004

Manoli, S., Coppola, S., Duranti, C., Lulli, M., Magni, L., Kuppalu, N., et al. (2019). The Activity of Kv 11.1 Potassium Channel Modulates F-Actin Organization During Cell Migration of Pancreatic Ductal Adenocarcinoma Cells. *Cancers (Basel)* 11 (2), 135. doi: 10.3390/cancers11020135

Marino, C. R., Matovcik, L. M., Gorelick, F. S., and Cohn, J. A. (1991). Localization of the cystic fibrosis transmembrane conductance regulator in pancreas. *J. Clin. Invest.* 88 (2), 712–716. doi: 10.1172/JCI115358

Marty, A., Tan, Y. P., and Trautmann, A. (1984). Three types of calcium-dependent channel in rat lacrimal glands. *J. Physiol.* 357, 293–325. doi: 10.1113/jphysiol.1984.sp015501

Maruyama, Y., Petersen, O. H., Flanagan, P., and Pearson, G. T. (1983). Quantification of Ca2+-activated K+ channels under hormonal control in pig pancreas acinar cells. *Nature* 305 (5931), 228–232. doi: 10.1038/305228a0

Matthews, E. K., Petersen, O. H., and Williams, J. A. (1973). Pancreatic acinar cells: acetylcholine-induced membrane depolarization, calcium efflux and amylase release. *J. Physiol.* 234 (3), 689–701. doi: 10.1113/jphysiol.1973.sp010367

Mazzone, A., Eisenman, S. T., Strege, P. R., Yao, Z., Ordog, T., Gibbons, S. J., et al. (2012). Inhibition of cell proliferation by a selective inhibitor of the Ca(2+)-activated Cl(-) channel, Ano1. *Biochem. Biophys. Res. Commun.* 427 (2), 248–253. doi: 10.1016/j.bbrc.2012.09.022

McDonald, F. J., Snyder, P. M., McCray, P. B.Jr., and Welsh, M. J. (1994). Cloning, expression, and tissue distribution of a human amiloride-sensitive Na+ channel. *Am. J. Physiol.* 266 (6 Pt 1), L728–L734. doi: 10.1152/ajplung.1994.266.6.L728

McDonald, T. V., Yu, Z., Ming, Z., Palma, E., Meyers, M. B., Wang, K. W., et al. (1997). A minK-HERG complex regulates the cardiac potassium current I(Kr). *Nature* 388 (6639), 289–292. doi: 10.1038/40882

McWilliams, R. R., Petersen, G. M., Rabe, K. G., Holtegaard, L. M., Lynch, P. J., Bishop, M. D., et al. (2010). Cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations and risk for pancreatic adenocarcinoma. *Cancer* 116 (1), 203–209. doi: 10.1002/cncr.24697

Miao, X., Liu, G., Xu, X., Xie, C., Sun, F., Yang, Y., et al. (2008). High expression of vanilloid receptor-1 is associated with better prognosis of patients with hepatocellular carcinoma. *Cancer Genet. Cytogenet.* 186 (1), 25–32. doi: 10.1016/j.cancergencyto.2008.05.011

Mo, P., and Yang, S. (2018). The store-operated calcium channels in cancer metastasis: from cell migration, invasion to metastatic colonization. *Front. Biosci. (Landmark Ed.)* 23, 1241–1256. doi: 10.2741/4641

Mogami, H., Nakano, K., Tepikin, A. V., and Petersen, O. H. (1997). Ca2+ flow via tunnels in polarized cells: recharging of apical Ca2+ stores by focal Ca2+ entry through basal membrane patch. *Cell* 88 (1), 49–55. doi: 10.1016/s0092-8674(00)81857-7

Morelli, M. B., Amantini, C., Nabissi, M., Liberati, S., Cardinali, C., Farfariello, V., et al. (2014). Cross-talk between alpha1D-adrenoceptors and transient receptor potential vanilloid type 1 triggers prostate cancer cell proliferation. *BMC Cancer* 14, 921. doi: 10.1186/1471-2407-14-921

Mu, D., Chen, L., Zhang, X., See, L. H., Koch, C. M., Yen, C., et al. (2003). Genomic amplification and oncogenic properties of the KCNK9 potassium channel gene. *Cancer Cell* 3 (3), 297–302. doi: 10.1016/s1535-6108(03)00054-0

Nagy, D., Gonczi, M., Dienes, B., Szoor, A., Fodor, J., Nagy, Z., et al. (2014). Silencing the KCNK9 potassium channel (TASK-3) gene disturbs mitochondrial function, causes mitochondrial depolarization, and induces apoptosis of human melanoma cells. *Arch. Dermatol. Res.* 306 (10), 885–902. doi: 10.1007/s00403-014-1511-5

Neglia, J. P., FitzSimmons, S. C., Maisonneuve, P., Schoni, M. H., Schoni-Affolter, F., Corey, M., et al. (1995). The risk of cancer among patients with cystic fibrosis. Cystic Fibrosis and Cancer Study Group. *N. Engl. J. Med.* 332 (8), 494–499. doi: 10.1056/NEJM199502233320803

Nguyen, T. D., and Moody, M. W. (1998). Calcium-activated potassium conductances on cultured nontransformed dog pancreatic duct epithelial cells. *Pancreas* 17 (4), 348–358. doi: 10.1097/00006676-199811000-00005

Nguyen, T. D., Koh, D. S., Moody, M. W., Fox, N. R., Savard, C. E., Kuver, R., et al. (1997). Characterization of two distinct chloride channels in cultured dog pancreatic duct epithelial cells. *Am. J. Physiol.* 272 (1 Pt 1), G172–G180. doi: 10.1152/ajpgi.1997.272.1.G172

Niemeyer, B. A., Mery, L., Zawar, C., Suckow, A., Monje, F., Pardo, L. A., et al. (2001). Ion channels in health and disease. 83rd Boehringer Ingelheim Fonds International Titisee Conference. *EMBO Rep.* 2 (7), 568–573. doi: 10.1093/embo-reports/kve145

North, W. G., Liu, F., Lin, L. Z., Tian, R., and Akerman, B. (2017). NMDA receptors are important regulators of pancreatic cancer and are potential targets for treatment. *Clin. Pharmacol.* 9, 79–86. doi: 10.2147/CPAA.S140057

Novak, I., and Hansen, M. R. (2002). Where have all the Na+ channels gone? In search of functional ENaC in exocrine pancreas. *Biochim. Biophys. Acta* 1566 (1-2), 162–168. doi: 10.1016/s0005-2736(02)00598-9

Novak, I., Wang, J., Henriksen, K. L., Haanes, K. A., Krabbe, S., Nitschke, R., et al. (2011). Pancreatic bicarbonate secretion involves two proton pumps. *J. Biol. Chem.* 286 (1), 280–289. doi: 10.1074/jbc.M110.136382

Novak, I., Haanes, K. A., and Wang, J. (2013). Acid-base transport in pancreas-new challenges. *Front. Physiol.* 4, 380. doi: 10.3389/fphys.2013.00380

Novak, I. (2000). Keeping up with bicarbonate. *J. Physiol.* 528 Pt 2, 235. doi: 10.1111/j.1469-7793.2000.00235.x

Ochi, A., Graffeo, C. S., Zambirinis, C. P., Rehman, A., Hackman, M., Fallon, N., et al. (2012). Toll-like receptor 7 regulates pancreatic carcinogenesis in mice and humans. *J. Clin. Invest.* 122 (11), 4118–4129. doi: 10.1172/JCI63606

Ohta, E., Itoh, T., Nemoto, T., Kumagai, J., Ko, S. B., Ishibashi, K., et al. (2009). Pancreas-specific aquaporin 12 null mice showed increased susceptibility to caerulein-induced acute pancreatitis. *Am. J. Physiol. Cell Physiol.* 297 (6), C1368–C1378. doi: 10.1152/ajpcell.00117.2009

Okeke, E., Parker, T., Dingsdale, H., Concannon, M., Awais, M., Voronina, S., et al. (2016). Epithelial-mesenchymal transition, IP3 receptors and ER-PM junctions: translocation of Ca2+ signalling complexes and regulation of migration. *Biochem. J.* 473 (6), 757–767. doi: 10.1042/BJ20150364

Ousingsawat, J., Martins, J. R., Schreiber, R., Rock, J. R., Harfe, B. D., and Kunzelmann, K. (2009). Loss of TMEM16A causes a defect in epithelial Ca2+-dependent chloride transport. *J. Biol. Chem.* 284 (42), 28698–28703. doi: 10.1074/jbc.M109.012120

Pallagi, P., Hegyi, P., and Rakonczay, Z. Jr. (2015). The Physiology and Pathophysiology of Pancreatic Ductal Secretion: The Background for Clinicians. *Pancreas* 44 (8), 1211–1233. doi: 10.1097/MPA.0000000000000421

Pandiri, A. R. (2014). Overview of exocrine pancreatic pathobiology. *Toxicol. Pathol.* 42 (1), 207–216. doi: 10.1177/0192623313509907

Papadopoulos, M. C., and Saadoun, S. (2015). Key roles of aquaporins in tumor biology. *Biochim. Biophys. Acta* 1848 (10 Pt B), 2576–2583. doi: 10.1016/j.bbamem.2014.09.001

Pardo, L. A., and Stuhmer, W. (2014). The roles of K(+) channels in cancer. *Nat. Rev. Cancer* 14 (1), 39–48. doi: 10.1038/nrc3635

Pardo, L. A., del Camino, D., Sanchez, A., Alves, F., Bruggemann, A., Beckh, S., et al. (1999). Oncogenic potential of EAG K(+) channels. *EMBO J.* 18 (20), 5540–5547. doi: 10.1093/emboj/18.20.5540

Park, M. K., Lomax, R. B., Tepikin, A. V., and Petersen, O. H. (2001). Local uncaging of caged Ca(2+) reveals distribution of Ca(2+)-activated Cl(-) channels in pancreatic acinar cells. *Proc. Natl. Acad. Sci. U. S. A.* 98 (19), 10948–10953. doi: 10.1073/pnas.181353798

Park, H. W., Nam, J. H., Kim, J. Y., Namkung, W., Yoon, J. S., Lee, J. S., et al. (2010). Dynamic regulation of CFTR bicarbonate permeability by [Cl-]i and its role in pancreatic bicarbonate secretion. *Gastroenterology* 139 (2), 620–631. doi: 10.1053/j.gastro.2010.04.004

Pascua, P., Garcia, M., Fernandez-Salazar, M. P., Hernandez-Lorenzo, M. P., Calvo, J. J., Colledge, W. H., et al. (2009). Ducts isolated from the pancreas of CFTR-null mice secrete fluid. *Pflugers Arch.* 459 (1), 203–214. doi: 10.1007/s00424-009-0704-9

Pearson, G. T., Flanagan, P. M., and Petersen, O. H. (1984). Neural and hormonal control of membrane conductance in the pig pancreatic acinar cell. *Am. J. Physiol.* 247 (5 Pt 1), G520–G526. doi: 10.1152/ajpgi.1984.247.5.G520

Pedersen, S. F., and Stock, C. (2013). Ion channels and transporters in cancer: pathophysiology, regulation, and clinical potential. *Cancer Res.* 73 (6), 1658–1661. doi: 10.1158/0008-5472.CAN-12-4188

Pedersen, S. F., Novak, I., Alves, F., Schwab, A., and Pardo, L. A. (2017). Alternating pH landscapes shape epithelial cancer initiation and progression: Focus on pancreatic cancer. *Bioessays* 39 (6). doi: 10.1002/bies.201600253

Peretti, M., Angelini, M., Savalli, N., Florio, T., Yuspa, S. H., and Mazzanti, M. (2015). Chloride channels in cancer: Focus on chloride intracellular channel 1 and 4 (CLIC1 AND CLIC4) proteins in tumor development and as novel therapeutic targets. *Biochim. Biophys. Acta* 1848 (10 Pt B), 2523–2531. doi: 10.1016/j.bbamem.2014.12.012

Pessia, M., Imbrici, P., D'Adamo, M. C., Salvatore, L., and Tucker, S. J. (2001). Differential pH sensitivity of Kir4.1 and Kir4.2 potassium channels and their modulation by heteropolymerisation with Kir5.1. *J. Physiol.* 532 (Pt 2), 359–367. doi: 10.1111/j.1469-7793.2001.0359f.x

Petersen, O. H., and Findlay, I. (1987). Electrophysiology of the pancreas. *Physiol. Rev.* 67 (3), 1054–1116. doi: 10.1152/physrev.1987.67.3.1054

Petersen, O. H., and Gallacher, D. V. (1988). Electrophysiology of pancreatic and salivary acinar cells. *Annu. Rev. Physiol.* 50, 65–80. doi: 10.1146/annurev.ph.50.030188.000433

Petersen, O. H., and Tepikin, A. V. (2008). Polarized calcium signaling in exocrine gland cells. *Annu. Rev. Physiol.* 70, 273–299. doi: 10.1146/annurev.physiol.70.113006.100618

Petersen, O. H., and Ueda, N. (1976). Pancreatic acinar cells: the role of calcium in stimulus-secretion coupling. *J. Physiol.* 254 (3), 583–606. doi: 10.1113/jphysiol.1976.sp011248

Petersen, O. H., Findlay, I., Iwatsuki, N., Singh, J., Gallacher, D. V., Fuller, C. M., et al. (1985). Human pancreatic acinar cells: studies of stimulus-secretion coupling. *Gastroenterology* 89 (1), 109–117. doi: 10.1016/0016-5085(85)90751-6

Petersen, O. H. (1992). Stimulus-secretion coupling: cytoplasmic calcium signals and the control of ion channels in exocrine acinar cells. *J. Physiol.* 448, 1–51. doi: 10.1113/jphysiol.1992.sp019028

Petersen, O. H. (2005). Ca2+ signalling and Ca2+-activated ion channels in exocrine acinar cells. *Cell Calcium* 38 (3-4), 171–200. doi: 10.1016/j.ceca.2005.06.024

Petersen, O. H. (2014). Calcium signalling and secretory epithelia. *Cell Calcium* 55 (6), 282–289. doi: 10.1016/j.ceca.2014.01.003

Pond, A. L., Scheve, B. K., Benedict, A. T., Petrecca, K., Van Wagoner, D. R., Shrier, A., et al. (2000). Expression of distinct ERG proteins in rat, mouse, and human heart. Relation to functional I(Kr) channels. *J. Biol. Chem.* 275 (8), 5997–6006. doi: 10.1074/jbc.275.8.5997

Premkumar, L. S., and Abooj, M. (2013). TRP channels and analgesia. *Life Sci.* 92 (8-9), 415–424. doi: 10.1016/j.lfs.2012.08.010

Prevarskaya, N., Zhang, L., and Barritt, G. (2007). TRP channels in cancer. *Biochim. Biophys. Acta* 1772 (8), 937–946. doi: 10.1016/j.bbadis.2007.05.006

Prevarskaya, N., Skryma, R., and Shuba, Y. (2010). Ion channels and the hallmarks of cancer. *Trends Mol. Med.* 16 (3), 107–121. doi: 10.1016/j.molmed.2010.01.005

Prevarskaya, N., Skryma, R., and Shuba, Y. (2018). Ion Channels in Cancer: Are Cancer Hallmarks Oncochannelopathies? *Physiol. Rev.* 98 (2), 559–621. doi: 10.1152/physrev.00044.2016

Radisky, D. C., and LaBarge, M. A. (2008). Epithelial-mesenchymal transition and the stem cell phenotype. *Cell Stem Cell* 2 (6), 511–512. doi: 10.1016/j.stem.2008.05.007

Ragsdale, D. S., Scheuer, T., and Catterall, W. A. (1991). Frequency and voltage-dependent inhibition of type IIA Na+ channels, expressed in a mammalian cell line, by local anesthetic, antiarrhythmic, and anticonvulsant drugs. *Mol. Pharmacol.* 40 (5), 756–765.

Randriamampita, C., Chanson, M., and Trautmann, A. (1988). Calcium and secretagogues-induced conductances in rat exocrine pancreas. *Pflugers Arch.* 411 (1), 53–57. doi: 10.1007/BF00581646

Reubi, J. C., Waser, B., Gugger, M., Friess, H., Kleeff, J., Kayed, H., et al. (2003). Distribution of CCK1 and CCK2 receptors in normal and diseased human pancreatic tissue. *Gastroenterology* 125 (1), 98–106. doi: 10.1016/s0016-5085(03)00697-8

Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., et al. (1989). Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* 245 (4922), 1066–1073. doi: 10.1126/science.2475911

Rodriguez-Boulan, E., and Nelson, W. J. (1989). Morphogenesis of the polarized epithelial cell phenotype. *Science* 245 (4919), 718–725. doi: 10.1126/science.2672330

Roux, B. (2017). Ion channels and ion selectivity. *Essays Biochem.* 61 (2), 201–209. doi: 10.1042/EBC20160074

Ruiz, C., Martins, J. R., Rudin, F., Schneider, S., Dietsche, T., Fischer, C. A., et al. (2012). Enhanced expression of ANO1 in head and neck squamous cell carcinoma causes cell migration and correlates with poor prognosis. *PloS One* 7 (8), e43265. doi: 10.1371/journal.pone.0043265

Rybarczyk, P., Gautier, M., Hague, F., Dhennin-Duthille, I., Chatelain, D., Kerr-Conte, J., et al. (2012). Transient receptor potential melastatin-related 7 channel is overexpressed in human pancreatic ductal adenocarcinomas and regulates human pancreatic cancer cell migration. *Int. J. Cancer* 131 (6), E851–E861. doi: 10.1002/ijc.27487

Rybarczyk, P., Vanlaeys, A., Brassart, B., Dhennin-Duthille, I., Chatelain, D., Sevestre, H., et al. (2017). The Transient Receptor Potential Melastatin 7

Channel Regulates Pancreatic Cancer Cell Invasion through the Hsp90alpha/uPA/MMP2 pathway. *Neoplasia* 19 (4), 288–300. doi: 10.1016/j.neo.2017.01.004

Sanchez, M. G., Sanchez, A. M., Collado, B., Malagarie-Cazenave, S., Olea, N., Carmena, M. J., et al. (2005). Expression of the transient receptor potential vanilloid 1 (TRPV1) in LNCaP and PC-3 prostate cancer cells and in human prostate tissue. *Eur. J. Pharmacol.* 515 (1-3), 20–27. doi: 10.1016/j.ejphar.2005.04.010

Sanguinetti, M. C., Jiang, C., Curran, M. E., and Keating, M. T. (1995). A mechanistic link between an inherited and an acquired cardiac arrhythmia: HERG encodes the IKr potassium channel. *Cell* 81 (2), 299–307. doi: 10.1016/0092-8674(95)90340-2

Sato, K., Ishizuka, J., Cooper, C. W., Chung, D. H., Tsuchiya, T., Uchida, T., et al. (1994). Inhibitory effect of calcium channel blockers on growth of pancreatic cancer cells. *Pancreas* 9 (2), 193–202. doi: 10.1097/00006676-199403000-00009

Sauter, D. R. P., Novak, I., Pedersen, S. F., Larsen, E. H., and Hoffmann, E. K. (2015). ANO1 (TMEM16A) in pancreatic ductal adenocarcinoma (PDAC). *Pflugers Arch.* 467 (7), 1495–1508. doi: 10.1007/s00424-014-1598-8

Sauter, D. R., Sorensen, C. E., Rapedius, M., Bruggemann, A., and Novak, I. (2016). pH-sensitive K(+) channel TREK-1 is a novel target in pancreatic cancer. *Biochim. Biophys. Acta* 1862 (10), 1994–2003. doi: 10.1016/j.bbadis.2016.07.009

Schneider, G., Siveke, J. T., Eckel, F., and Schmid, R. M. (2005). Pancreatic cancer: basic and clinical aspects. *Gastroenterology* 128 (6), 1606–1625. doi: 10.1053/j.gastro.2005.04.001

Schroeder, B. C., Cheng, T., Jan, Y. N., and Jan, L. Y. (2008). Expression cloning of TMEM16A as a calcium-activated chloride channel subunit. *Cell* 134 (6), 1019–1029. doi: 10.1016/j.cell.2008.09.003

Serrano-Novillo, C., Capera, J., Colomer-Molera, M., Condom, E., Ferreres, J. C., and Felipe, A. (2019). Implication of Voltage-Gated Potassium Channels in Neoplastic Cell Proliferation. *Cancers (Basel)* 11 (3), 287. doi: 10.3390/cancers11030287

Sette, A., Spadavecchia, J., Landoulsi, J., Casale, S., Haye, B., Crociani, O., et al. (2013). Development of novel anti-Kv 11.1 antibody-conjugated PEG-TiO2 nanoparticles for targeting pancreatic ductal adenocarcinoma cells. *J. Nanopart Res.* 15, 2111. doi: 10.1007/s11051-013-2111-6

Shapovalov, G., Ritaine, A., Skryma, R., and Prevarskaya, N. (2016). Role of TRP ion channels in cancer and tumorigenesis. *Semin. Immunopathol.* 38 (3), 357–369. doi: 10.1007/s00281-015-0525-1

Sheldon, C. D., Hodson, M. E., Carpenter, L. M., and Swerdlow, A. J. (1993). A cohort study of cystic fibrosis and malignancy. *Br. J. Cancer* 68 (5), 1025–1028. doi: 10.1038/bjc.1993.474

Shen, S., Gui, T., and Ma, C. (2017). Identification of molecular biomarkers for pancreatic cancer with mRMR shortest path method. *Oncotarget* 8 (25), 41432–41439. doi: 10.18632/oncotarget.18186

Shuck, M. E., Piser, T. M., Bock, J. H., Slightom, J. L., Lee, K. S., and Bienkowski, M. J. (1997). Cloning and characterization of two K+ inward rectifier (Kir) 1.1 potassium channel homologs from human kidney (Kir1.2 and Kir1.3). *J. Biol. Chem.* 272 (1), 586–593. doi: 10.1074/jbc.272.1.586

Singh, A. P., Chauhan, S. C., Andrianifahanana, M., Moniaux, N., Meza, J. L., Copin, M. C., et al. (2007). MUC4 expression is regulated by cystic fibrosis transmembrane conductance regulator in pancreatic adenocarcinoma cells via transcriptional and post-translational mechanisms. *Oncogene* 26 (1), 30–41. doi: 10.1038/sj.onc.1209764

Song, H., Dong, M., Zhou, J., Sheng, W., Li, X., and Gao, W. (2018). Expression and prognostic significance of TRPV6 in the development and progression of pancreatic cancer. *Oncol. Rep.* 39 (3), 1432–1440. doi: 10.3892/or.2018.6216

Song, H. Y., Zhou, L., Hou, X. F., and Lian, H. (2019). Anoctamin 5 regulates cell proliferation and migration in pancreatic cancer. *Int. J. Clin. Exp. Pathol.* 12 (12), 4263–4270.

Steward, M. C., Ishiguro, H., and Case, R. M. (2005). Mechanisms of bicarbonate secretion in the pancreatic duct. *Annu. Rev. Physiol.* 67, 377–409. doi: 10.1146/annurev.physiol.67.031103.153247

Stock, C., and Schwab, A. (2015). Ion channels and transporters in metastasis. *Biochim. Biophys. Acta* 1848 (10 Pt B), 2638–2646. doi: 10.1016/j.bbamem.2014.11.012

Stoklosa, P., Borgstrom, A., Kappel, S., and Peinelt, C. (2020). TRP Channels in Digestive Tract Cancers. *Int. J. Mol. Sci.* 21 (5), 1877. doi: 10.3390/ijms21051877

Suzuki, K., Petersen, C. C., and Petersen, O. H. (1985). Hormonal activation of single K+ channels via internal messenger in isolated pancreatic acinar cells. *FEBS Lett.* 192 (2), 307–312. doi: 10.1016/0014-5793(85)80131-9

Tawfik, D., Zaccagnino, A., Bernt, A., Szczepanowski, M., Klapper, W., Schwab, A., et al. (2020). The A818-6 system as an in-vitro model for studying the role of the transportome in pancreatic cancer. *BMC Cancer* 20 (1), 264. doi: 10.1186/s12885-020-06773-w

Teisseyre, A., Palko-Labuz, A., Sroda-Pomianek, K., and Michalak, K. (2019). Voltage-Gated Potassium Channel Kv1.3 as a Target in Therapy of Cancer. *Front. Oncol.* 9, 933. doi: 10.3389/fonc.2019.00933

Thevenod, F. (2002). Ion channels in secretory granules of the pancreas and their role in exocytosis and release of secretory proteins. *Am. J. Physiol. Cell Physiol.* 283 (3), C651–C672. doi: 10.1152/ajpcell.00600.2001

Thompson-Vest, N., Shimizu, Y., Hunne, B., and Furness, J. B. (2006). The distribution of intermediate-conductance, calcium-activated, potassium (IK) channels in epithelial cells. *J. Anat.* 208 (2), 219–229. doi: 10.1111/j.1469-7580.2006.00515.x

Tolon, R. M., Sanchez-Franco, F., Lopez Fernandez, J., Lorenzo, M. J., Vazquez, G. F., and Cacicedo, L. (1996). Regulation of somatostatin gene expression by veratridine-induced depolarization in cultured fetal cerebrocortical cells. *Brain Res. Mol. Brain Res.* 35 (1-2), 103–110. doi: 10.1016/0169-328x(95)00188-x

Ulareanu, R., Chiritoiu, G., Cojocaru, F., Deftu, A., Ristoiu, V., Stanica, L., et al. (2017). N-glycosylation of the transient receptor potential melastatin 8 channel is altered in pancreatic cancer cells. *Tumour Biol.* 39 (8):1010428317720940. doi: 10.1177/1010428317720940

Venglovecz, V., Hegyi, P., Rakonczay, Z.Jr., Tiszlavicz, L., Nardi, A., Grunnet, M., et al. (2011). Pathophysiological relevance of apical large-conductance Ca(2)+-activated potassium channels in pancreatic duct epithelial cells. *Gut* 60 (3), 361–369. doi: 10.1136/gut.2010.214213

Venglovecz, V., Rakonczay, Z.Jr., Gray, M. A., and Hegyi, P. (2015). Potassium channels in pancreatic duct epithelial cells: their role, function and pathophysiological relevance. *Pflugers Arch.* 467 (4), 625–640. doi: 10.1007/s00424-014-1585-0

Venglovecz, V., Pallagi, P., Kemeny, L. V., Balazs, A., Balla, Z., Becskehazi, E., et al. (2018). The Importance of Aquaporin 1 in Pancreatitis and Its Relation to the CFTR Cl(-) Channel. *Front. Physiol.* 9, 854. doi: 10.3389/fphys.2018.00854

Vercelli, C., Barbero, R., Cuniberti, B., Racca, S., Abbadessa, G., Piccione, F., et al. (2014). Transient receptor potential vanilloid 1 expression and functionality in mcf-7 cells: a preliminary investigation. *J. Breast Cancer* 17 (4), 332–338. doi: 10.4048/jbc.2014.17.4.332

Voloshyna, I., Besana, A., Castillo, M., Matos, T., Weinstein, I. B., Mansukhani, M., et al. (2008). TREK-1 is a novel molecular target in prostate cancer. *Cancer Res.* 68 (4), 1197–1203. doi: 10.1158/0008-5472.CAN-07-5163

Waldmann, R., Champigny, G., Bassilana, F., Voilley, N., and Lazdunski, M. (1995). Molecular cloning and functional expression of a novel amiloride-sensitive Na+ channel. *J. Biol. Chem.* 270 (46), 27411–27414. doi: 10.1074/jbc.270.46.27411

Wang, B. J., and Cui, Z. J. (2007). How does cholecystokinin stimulate exocrine pancreatic secretion? From birds, rodents, to humans. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 292 (2), R666–R678. doi: 10.1152/ajpregu.00131.2006

Wang, J., and Novak, I. (2013). Ion transport in human pancreatic duct epithelium, Capan-1 cells, is regulated by secretin, VIP, acetylcholine, and purinergic receptors. *Pancreas* 42 (3), 452–460. doi: 10.1097/MPA.0b013e318264c302

Wang, J., Haanes, K. A., and Novak, I. (2013). Purinergic regulation of CFTR and Ca(2+)-activated Cl(-) channels and K(+) channels in human pancreatic duct epithelium. *Am. J. Physiol. Cell Physiol.* 304 (7), C673–C684. doi: 10.1152/ajpcell.00196.2012

Wang, F., Jin, R., Zou, B. B., Li, L., Cheng, F. W., Luo, X., et al. (2016). Activation of Toll-like receptor 7 regulates the expression of IFN-lambda1, p53, PTEN, VEGF, TIMP-1 and MMP-9 in pancreatic cancer cells. *Mol. Med. Rep.* 13 (2), 1807–1812. doi: 10.3892/mmr.2015.4730

Wang, J., Shen, J., Zhao, K., Hu, J., Dong, J., and Sun, J. (2019). STIM1 overexpression in hypoxia microenvironment contributes to pancreatic

carcinoma progression. *Cancer Biol. Med.* 16 (1), 100–108. doi: 10.20892/j.issn.2095-3941.2018.0304

Warth, R., and Barhanin, J. (2002). The multifaceted phenotype of the knockout mouse for the KCNE1 potassium channel gene. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 282 (3), R639–R648. doi: 10.1152/ajpregu.00649.2001

Warth, R., Garcia Alzamora, M., Kim, J. K., Zdebik, A., Nitschke, R., Bleich, M., et al. (2002). The role of KCNQ1/KCNE1 K(+) channels in intestine and pancreas: lessons from the KCNE1 knockout mouse. *Pflugers Arch.* 443 (5-6), 822–828. doi: 10.1007/s00424-001-0751-3

Wen, L., Voronina, S., Javed, M. A., Awais, M., Szatmary, P., Latawiec, D., et al. (2015). Inhibitors of ORAI1 Prevent Cytosolic Calcium-Associated Injury of Human Pancreatic Acinar Cells and Acute Pancreatitis in 3 Mouse Models. *Gastroenterology* 149481-492 (2), e487. doi: 10.1053/j.gastro.2015.04.015

Williams, S., Bateman, A., and O'Kelly, I. (2013). Altered expression of two-pore domain potassium (K2P) channels in cancer. *PloS One* 8 (10), e74589. doi: 10.1371/journal.pone.0074589

Wilschanski, M., and Novak, I. (2013). The cystic fibrosis of exocrine pancreas. *Cold Spring Harb. Perspect. Med.* 3 (5):a009746. doi: 10.1101/cshperspect.a009746

Winpenny, J. P., Verdon, B., McAlroy, H. L., Colledge, W. H., Ratcliff, R., Evans, M. J., et al. (1995). Calcium-activated chloride conductance is not increased in pancreatic duct cells of CF mice. *Pflugers Arch.* 430 (1), 26–33. doi: 10.1007/BF00373836

Worley, P. F., Zeng, W., Huang, G. N., Yuan, J. P., Kim, J. Y., Lee, M. G., et al. (2007). TRPC channels as STIM1-regulated store-operated channels. *Cell Calcium* 42 (2), 205–211. doi: 10.1016/j.ceca.2007.03.004

Wright, A. M., Gong, X., Verdon, B., Linsdell, P., Mehta, A., Riordan, J. R., et al. (2004). Novel regulation of cystic fibrosis transmembrane conductance regulator (CFTR) channel gating by external chloride. *J. Biol. Chem.* 279 (40), 41658–41663. doi: 10.1074/jbc.M405517200

Yamaguchi, M., Steward, M. C., Smallbone, K., Sohma, Y., Yamamoto, A., Ko, S. B., et al. (2017). Bicarbonate-rich fluid secretion predicted by a computational model of guinea-pig pancreatic duct epithelium. *J. Physiol.* 595 (6), 1947–1972. doi: 10.1113/JP273306

Yang, B., Song, Y., Zhao, D., and Verkman, A. S. (2005). Phenotype analysis of aquaporin-8 null mice. *Am. J. Physiol. Cell Physiol.* 288 (5), C1161–C1170. doi: 10.1152/ajpcell.00564.2004

Yang, Y. D., Cho, H., Koo, J. Y., Tak, M. H., Cho, Y., Shim, W. S., et al. (2008). TMEM16A confers receptor-activated calcium-dependent chloride conductance. *Nature* 455 (7217), 1210–1215. doi: 10.1038/nature07313

Yee, N. S., Zhou, W., and Lee, M. (2010). Transient receptor potential channel TRPM8 is over-expressed and required for cellular proliferation in pancreatic adenocarcinoma. *Cancer Lett.* 297 (1), 49–55. doi: 10.1016/j.canlet.2010.04.023

Yee, N. S., Zhou, W., and Liang, I. C. (2011). Transient receptor potential ion channel Trpm7 regulates exocrine pancreatic epithelial proliferation by Mg2+-sensitive Socs3a signaling in development and cancer. *Dis. Model Mech.* 4 (2), 240–254. doi: 10.1242/dmm.004564

Yee, N. S., Chan, A. S., Yee, J. D., and Yee, R. K. (2012a). TRPM7 and TRPM8 Ion Channels in Pancreatic Adenocarcinoma: Potential Roles as Cancer Biomarkers and Targets. *Sci. (Cairo)* 2012, 415158. doi: 10.6064/2012/415158

Yee, N. S., Zhou, W., Lee, M., and Yee, R. K. (2012b). Targeted silencing of TRPM7 ion channel induces replicative senescence and produces enhanced cytotoxicity with gemcitabine in pancreatic adenocarcinoma. *Cancer Lett.* 318 (1), 99–105. doi: 10.1016/j.canlet.2011.12.007

Yee, N. S., Li, Q., Kazi, A. A., Yang, Z., Berg, A., and Yee, R. K. (2014). Aberrantly Over-Expressed TRPM8 Channels in Pancreatic Adenocarcinoma: Correlation with Tumor Size/Stage and Requirement for Cancer Cells Invasion. *Cells* 3 (2), 500–516. doi: 10.3390/cells3020500

Yee, N. S., Kazi, A. A., Li, Q., Yang, Z., Berg, A., and Yee, R. K. (2015). Aberrant over-expression of TRPM7 ion channels in pancreatic cancer: required for cancer cell invasion and implicated in tumor growth and metastasis. *Biol. Open* 4 (4), 507–514. doi: 10.1242/bio.20137088

Yokoyama, T., Takemoto, M., Hirakawa, M., and Saino, T. (2019). Different immunohistochemical localization for TMEM16A and CFTR in acinar and ductal cells of rat major salivary glands and exocrine pancreas. *Acta Histochem.* 121 (1), 50–55. doi: 10.1016/j.acthis.2018.10.013

Yurtsever, Z., Sala-Rabanal, M., Randolph, D. T., Scheaffer, S. M., Roswit, W. T., Alevy, Y. G., et al. (2012). Self-cleavage of human CLCA1 protein by a novel internal metalloprotease domain controls calcium-activated chloride channel activation. *J. Biol. Chem.* 287 (50), 42138–42149. doi: 10.1074/jbc.M112.410282

Zaccagnino, A., Pilarsky, C., Tawfik, D., Sebens, S., Trauzold, A., Novak, I., et al. (2016). In silico analysis of the transportome in human pancreatic ductal adenocarcinoma. *Eur. Biophys. J.* 45 (7), 749–763. doi: 10.1007/s00249-016-1171-9

Zaccagnino, A., Manago, A., Leanza, L., Gontarewitz, A., Linder, B., Azzolini, M., et al. (2017). Tumor-reducing effect of the clinically used drug clofazimine in a SCID mouse model of pancreatic ductal adenocarcinoma. *Oncotarget* 8 (24), 38276–38293. doi: 10.18632/oncotarget.11299

Zdebik, A., Hug, M. J., and Greger, R. (1997). Chloride channels in the luminal membrane of rat pancreatic acini. *Pflugers Arch.* 434 (2), 188–194. doi: 10.1007/s004240050382

Zeiher, B. G., Eichwald, E., Zabner, J., Smith, J. J., Puga, A. P., McCray, P. B.Jr., et al. (1995). A mouse model for the delta F508 allele of cystic fibrosis. *J. Clin. Invest.* 96 (4), 2051–2064. doi: 10.1172/JCI118253

Zeng, W., Lee, M. G., Yan, M., Diaz, J., Benjamin, I., Marino, C. R., et al. (1997). Immuno and functional characterization of CFTR in submandibular and pancreatic acinar and duct cells. *Am. J. Physiol.* 273 (2 Pt 1), C442–C455. doi: 10.1152/ajpcell.1997.273.2.C442

Zhou, B., Irwanto, A., Guo, Y. M., Bei, J. X., Wu, Q., Chen, G., et al. (2012). Exome sequencing and digital PCR analyses reveal novel mutated genes related to the metastasis of pancreatic ductal adenocarcinoma. *Cancer Biol. Ther.* 13 (10), 871–879. doi: 10.4161/cbt.20839

Zhou, J., Zhang, L., Zheng, H., Ge, W., Huang, Y., Yan, Y., et al. (2020). Identification of chemoresistance-related mRNAs based on gemcitabine-resistant pancreatic cancer cell lines. *Cancer Med.* 9 (3), 1115–1130. doi: 10.1002/cam4.2764

Zhu, S., Zhou, H. Y., Deng, S. C., Deng, S. J., He, C., Li, X., et al. (2017). ASIC1 and ASIC3 contribute to acidity-induced EMT of pancreatic cancer through activating Ca(2+)/RhoA pathway. *Cell Death Dis.* 8 (5), e2806. doi: 10.1038/cddis.2017.189

Zou, Q., Yang, Z., Li, D., Liu, Z., and Yuan, Y. (2016). Association of chloride intracellular channel 4 and Indian hedgehog proteins with survival of patients with pancreatic ductal adenocarcinoma. *Int. J. Exp. Pathol.* 97 (6), 422–429. doi: 10.1111/iep.12213

Zou, W., Yang, Z., Li, D., Liu, Z., Zou, Q., and Yuan, Y. (2019). AQP1 and AQP3 Expression are Associated With Severe Symptoms and Poor-prognosis of the Pancreatic Ductal Adenocarcinoma. *Appl. Immunohistochem. Mol. Morphol.* 27 (1), 40–47. doi: 10.1097/PAI.0000000000000523

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 Schnipper, Dhennin-Duthille, Ahidouch and Ouadid-Ahidouch. 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 Pharmacology

REVIEW
published: 19 January 2021
doi: 10.3389/fphar.2020.586599

Ion Channels Orchestrate Pancreatic Ductal Adenocarcinoma Progression and Therapy

Verena Hofschröer[1], Karolina Najder[1], Micol Rugi[1], Rayhana Bouazzi[2], Marco Cozzolino[3], Annarosa Arcangeli[2], Gyorgy Panyi[3] and Albrecht Schwab[1]*

[1]Institute of Physiology II, University of Münster, Münster, Germany, [2]Department of Experimental and Clinical Medicine, Section of Internal Medicine, University of Florence, Florence, Italy, [3]Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

Pancreatic ductal adenocarcinoma is a devastating disease with a dismal prognosis. Therapeutic interventions are largely ineffective. A better understanding of the pathophysiology is required. Ion channels contribute substantially to the "hallmarks of cancer." Their expression is dysregulated in cancer, and they are "misused" to drive cancer progression, but the underlying mechanisms are unclear. Ion channels are located in the cell membrane at the interface between the intracellular and extracellular space. They sense and modify the tumor microenvironment which in itself is a driver of PDAC aggressiveness. Ion channels detect, for example, locally altered proton and electrolyte concentrations or mechanical stimuli and transduce signals triggered by these microenvironmental cues through association with intracellular signaling cascades. While these concepts have been firmly established for other cancers, evidence has emerged only recently that ion channels are drivers of PDAC aggressiveness. Particularly, they appear to contribute to two of the characteristic PDAC features: the massive fibrosis of the tumor stroma (desmoplasia) and the efficient immune evasion. Our critical review of the literature clearly shows that there is still a remarkable lack of knowledge with respect to the contribution of ion channels to these two typical PDAC properties. Yet, we can draw parallels from ion channel research in other fibrotic and inflammatory diseases. Evidence is accumulating that pancreatic stellate cells express the same "profibrotic" ion channels. Similarly, it is at least in part known which major ion channels are expressed in those innate and adaptive immune cells that populate the PDAC microenvironment. We explore potential therapeutic avenues derived thereof. Since drugs targeting PDAC-relevant ion channels are already in clinical use, we propose to repurpose those in PDAC. The quest for ion channel targets is both motivated and complicated by the fact that some of the relevant channels, for example, $K_{Ca}3.1$, are functionally expressed in the cancer, stroma, and immune cells. Only *in vivo* studies will reveal which arm of the balance we should put our weights on when developing channel-targeting PDAC therapies. The time is up to explore the efficacy of ion channel targeting in (transgenic) murine PDAC models before launching clinical trials with repurposed drugs.

Keywords: pancreatic ductal adenocarcinoma, ion channels, therapy, immune cells, fibrosis

OPEN ACCESS

Edited by:
Sébastien Roger, Université de Tours, France

Reviewed by:
Raphael Rapetti-Mauss, INSERM U1091 Institut de biologie de Valrose, France
Andrea J. Yool, University of Adelaide, Australia

****Correspondence:***
Verena Hofschröer
verena.hofschroeer@uni-muenster.de

Specialty section:
This article was submitted to Pharmacology of Ion Channels and Channelopathies, a section of the journal Frontiers in Pharmacology

Received: *23 July 2020*
Accepted: *30 October 2020*
Published: *19 January 2021*

Citation:
Hofschröer V, Najder K, Rugi M, Bouazzi R, Cozzolino M, Arcangeli A, Panyi G and Schwab A (2021) Ion Channels Orchestrate Pancreatic Ductal Adenocarcinoma Progression and Therapy. Front. Pharmacol. 11:586599. doi: 10.3389/fphar.2020.586599

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) progresses rapidly once fully developed and can easily overcome current treatment strategies. The aggressiveness of the disease leads to a disastrous outcome for patients. The current 5-year survival rate is still less than 10% (Rawla et al., 2019). This poor prognosis is due to the absence of clinical symptoms in the early stage combined with the characteristic properties of PDAC: desmoplasia, early local invasion and metastasis, immune evasion, and resistance to radio- and chemotherapy. Desmoplasia involves a strong reaction of the tumor stroma in which matrix-producing pancreatic stellate cells (PSCs) play a central role. Mutual stimulation of tumor, stroma, and immune cells leads, in a positive feedback cycle, to enhanced growth factor and matrix production, creating a microenvironment conducive to tumor growth, migration/invasion of tumor cells into the tumor stroma, and metastasis (Mahadevan and Von Hoff, 2007; Sperb et al., 2020). Despite knowledge of many molecular details of PDAC, the therapeutic benefit derived thereof has been disappointingly small. Thus, there is an urgent need for novel concepts and therapeutic targets for the treatment of PDAC.

A promising novel concept is the targeting of ion channels in cancer. They are a major class of membrane proteins that have the ability to sense and modify properties of the tumor microenvironment and transduce signaling cascades triggered by its constituents. Therefore, they play central roles in signaling within and among tumor and stromal cells as well as in the coupling of extracellular events with cellular responses (Djamgoz et al., 2014). Ion channels are expressed in every cell where they exert cell-specific functions and housekeeping functions such as generating the membrane potential which, in turn, is a prerequisite for many processes such as Ca^{2+} signaling. Being the "working horses" of epithelial cells, ion channels are also essential for the normal function of the exocrine pancreas (e.g., Hayashi and Novak, 2013; Wang et al., 2013). Conversely, ion channel mutations cause hereditary diseases, so-called channelopathies. The most frequent one in Caucasians is the mutation of the cystic fibrosis transmembrane conductance regulator, CFTR, which causes, among others, a hereditary chronic pancreatitis which is a risk factor for developing PDAC (Becker et al., 2014).

Reviews from recent years on the role of ion channels in cancer (Djamgoz et al., 2014; Klumpp et al., 2018; Prevarskaya et al., 2018; Bulk et al., 2020; Ling and Kalthoff, 2020; Schnipper et al., 2020) predominantly focused on how ion channels shape the aggressive cancer cell behavior. However, cancer must be viewed as a complex tissue composed of different cell types. This is particularly relevant for PDAC: PSCs and immune cells are deeply involved in PDAC pathophysiology. PSCs have an important share in creating a tumor microenvironment in PDAC that contributes to immune evasion and thereby to the aggressiveness and therapy refractoriness of the disease (Wang et al., 2020b; Hessmann et al., 2020; Sperb et al., 2020). So far, there is still a considerable lack of knowledge on how ion channels and the (ionic) tumor microenvironment contribute to these aspects of PDAC pathophysiology. Yet, it has become evident that the function of ion channels in noncancerous cells has to be considered, too. Recent work in prostate cancer has started to go into this direction (Farfariello et al., 2020).

This review will put a special emphasis on the role of ion channels in stromal and immune cells. We will propose concepts on how fibrosis and immune evasion could be addressed in PDAC therapy by ion channel targeting. Due to their location in the plasma membrane, ion channels are easily accessible and well-characterized. Drugs targeting those have been in clinical use since decades. This is clearly exemplified by Na^+ channel blockers acting as local anesthetics, antiarrhythmics, anticonvulsants, and diuretics. Moreover, there are drugs whose side effects involve ion channel blockade (e.g., $K_V11.1$ blockade by haloperidol, fluoxetine, tamoxifen and amitriptyline (Pointer et al., 2017) or $K_V10.1$ blockade by astemizole or imipramine (García-Ferreiro et al., 2004)). Other channel-targeting drugs such as the $K_{Ca}3.1$ blocker senicapoc have gone through phase III clinical trials (Ataga et al., 2011). The availability of such drugs provides us with an enormous advantage as they may be repurposed within the cancer treatment context (Kale et al., 2015). While developing a new drug "from scratch" takes on average 12 years and costs one billion dollars, repurposing requires only 2–3 years and 10 million dollars (Zheng et al., 2013).

REGULATION OF PANCREATIC CANCER CELL BEHAVIOR BY ION CHANNELS

K^+ Channels in Pancreatic Cancer Cells

By controlling the flow of potassium ions across the cell membrane, K^+ channels regulate a multitude of processes, both in healthy and pathological conditions, including cancer (Huang and Jan, 2014). In proliferating cells, such as cancer cells, the K^+ efflux mediated by K^+ channels modulates cancer cell behavior by 1) providing the electrochemical force needed for the influx of Ca^{2+} (e.g., through store-operated Ca^{2+} channels (Feske et al., 2015)), which is known to be important for G_0/G_1 and G_1/S transitions; 2) by transiently hyperpolarizing the membrane potential, which is also an important feature for cell cycle progression (Urrego et al., 2014); or by 3) being involved in cell volume regulation that highly relies on K^+ efflux (Hoffmann et al., 2009). K^+ channels may also work in a nonconductive manner by promoting signal transduction pathways involved in cell proliferation through interaction with other membrane proteins such as integrins (see below) (Becchetti et al., 2019).

Voltage-gated K^+ channels (K_V channels) are a large family of 40 genes grouped into 12 subfamilies. One of their key functions is the repolarization of the cell membrane potential of excitable cells (Wulff et al., 2009; Arcangeli and Becchetti, 2017). However, they are also found in nonexcitable cells where K_V channels play important roles in cell proliferation, Ca^{2+} signaling, migration, and cell volume regulation. Moreover, they promote cancer progression (Huang and Jan, 2014). The involvement of ion channels in PDAC, such as K_V channels, is summarized in **Table 1**.

$K_V1.3$ channels: In healthy humans, the $K_V1.3$ channels are mainly expressed in the central nervous system and in immune

TABLE 1 | Ion channel expression and their functional role in pancreatic cancer and stellate cells.

Channel	Function	Reference
K^+ channels		
$K_{Ca}3.1$	Functional expression in PDAC cell lines and elevated expression in PDAC tissue; cell proliferation	Jäger et al. (2004)
	Functional expression in PDAC cells; cell migration, proliferation, and invasion	Bonito et al. (2016)
	Subset of PDAC cell lines: Oxygen consumption, ATP production, and cellular proliferation	Kovalenko et al. (2016)
	Expression in PSC; migration and chemotaxis, $[Ca^{2+}]_i$ signaling, calpain activity, functional cooperation with TRPC3	Storck et al. (2017)
	High expression correlates with poor patient prognosis in PDAC	Zaccagnino et al. (2016); Jiang et al. (2017)
	Macrophage infiltration into cancer tissue via a Ca^{2+}-dependent activation of CXCL5-CCL20 secretion by PDAC cells	Jiang et al. (2019)
$K_{ir}3.1$	Highly expressed in PDAC	Brevet et al. (2009).
$K_{2P}1.1$ (TWIK-1)	mRNA up-regulation in PDAC tissue	Williams et al. (2013).
$K_{2P}2.1$ (TREK-1)	Expressed in PDAC cells (BxPC-3) Mediates pH-sensitive K^+ current Modulates the membrane potential (V_m)	Sauter et al. (2016).
	PSCs: mRNA expression	Fels et al. (2016)
$K_{2P}3.1$ (TASK-1)	mRNA down-regulation in tissues from PDAC patients	Williams et al. (2013)
$K_V1.3$	Reduced primary tumor weight *in vivo* by inhibitor clofazimine	Zaccagnino et al. (2017)
	Decreased expression in PDAC, associated with metastatic tumors	Brevet et al. (2009)
Mitochondrial channel mito$K_V1.3$	Apoptosis of cancer cells, cancer development, and progression in mouse models of PDAC	Leanza et al. (2017) and Zaccagnino et al. (2017)
$K_V10.1$ (hEAG)	Inhibition of channel activity by monoclonal antibodies; inhibition of tumor cell growth in mouse xenograft model of pancreatic cancer	Gómez-Varela et al. (2007)
$K_V11.1$ (hERG)	Expression in PDAC samples	Zhou et al. (2012)
	Cell growth and invasiveness	Feng et al. (2014)
	PDAC malignancy *in vitro* and *in vivo*; diagnostic and prognostic biomarker	Lastraioli et al. (2015)
	PDAC cell migration, modulator of f-actin organization, and Ca^{2+} signaling	Manoli et al. (2019)
TRP channels		
TRPC1	TGF-β stimulated Ca^{2+}-responses; migration and invasion (BxPc3 cells)	Dong et al. (2010)
	Mechanosignaling of murine PSC, pressure-dependent PSC activation	Fels et al. (2016)
TRPC3	Up-regulated in PDAC stroma; functional cooperation with $K_{Ca}3.1$; PSC migration and chemotaxis; and Ca^{2+} signaling	Storck et al. (2017)
TRPC6	PSCs: Cell migration, Ca^{2+} signaling, and cytokine secretion in hypoxia	Nielsen et al. (2017)
TRPM2	SIRT6-elevated ADPr levels increase TRPM2 activation; migration (BxPc3 cells)	Bauer et al. (2012) and Lin et al. (2018)
TRPM7	Overexpressed in PDAC tissue; correlated with poor patient survival	Rybarczyk et al. (2012)
	Overexpression correlates with increased tumor size and advanced tumor stages	Yee et al. (2015)
	PDAC cell invasion in Panc-1/MiaPaCa2; expression in lymph node metastasis and primary tumor correlation in human PDAC	Rybarczyk et al. (2017)
TRPM8	Up-regulated in PDAC cell lines and tissue; cell proliferation	Yee et al. (2010)
	Functional expression in the plasma membrane; cell migration (Panc-1 cells)	Cucu et al. (2014)
TRPV1	Overexpressed in PDAC and the involved neurons; potential link to pain intensity reported by cancer patients	Hartel et al. (2006)
TRPV4	Prolonged high fat/alcohol exposure increases TRPV4 expression in PSCs, fibrosis	Zhang et al. (2013)
	Pressure-modulated mRNA expression in PSCs	Fels et al. (2016)
TRPV6	Up-regulated in pancreatic cancer tissue; affects proliferation, migration, invasion, and apoptosis in PDAC	Song et al. (2018)
	Down-regulated in PDAC cell line and in the tumor epithelium of PDAC tissue	Zaccagnino et al. (2016) and Tawfik et al. (2020)
	Loss of function variants linked to early onset chronic pancreatitis (a risk factor for PDAC development)	Masamune et al. (2020)
Other ion channels		
ASICs		
ASIC1, ASIC3	Functional (over-)expression in PDAC; $[Ca^{2+}]_i$ signaling, EMT, liver and lung metastasis	Zhu et al. (2017)
P2X receptors		
P2X7	PSC proliferation and death	Haanes et al. (2012)
	Overexpressed in PDAC cell lines; cell survival, migration, and invasion	Giannuzzo et al. (2015)
	Tumor growth; PSC number/activity, fibrosis	Giannuzzo et al. (2016)
Piezo1	High mRNA levels in PSCs	Fels et al. (2016)
	PSCs: Ca^{2+} influx, cytoskeletal architecture, cell invasion, pH-dependent mechanosensation	Kuntze et al. (2020)
ORAI1/STIM1	Prosurvival antiapoptotic role by mediating store-operated Ca^{2+} entry	Kondratska et al. (2014)
CaCC (TMEM16A)	Functionally overexpressed in human PDAC cells; supports migration, but not proliferation	Sauter et al. (2015)
	Promotes pathogenesis of acute pancreatitis via IP_3R/Ca^{2+}/NFκB/IL-6 signaling	Wang et al. (2020a)
	Essential for EGF-induced store-operated Ca^{2+} entry during pancreatic cancer cell migration; overexpression correlates with low patient survival probability	Crottès et al. (2019)

cells (Cahalan and Chandy, 2009). $K_V1.3$ channel expression is found up-regulated in several PDAC cell lines (Zaccagnino et al., 2017). Overexpression of the channel is an advantage for cancer cells to promote proliferation and cell survival. This was illustrated by targeting PDAC cells with a $K_V1.3$ inhibitor (clofazimine) which induces apoptosis and reduces the weight of tumors grown from orthotopically transplanted PDAC cells (Zaccagnino et al., 2017). $K_V1.3$ is also expressed in the mitochondria (mito$K_V1.3$), where it regulates apoptosis in PDAC cells (Leanza et al., 2017). The above-cited data from PDAC cell lines, however, differ from those obtained in tissue samples from PDAC patients where $K_V1.3$ expression is down-regulated. This down-regulation correlates with metastasis. The diminished expression of $K_V1.3$ was explained as a result of the methylation of its promoter (Brevet et al., 2009).

$K_V10.1$ and $K_V11.1$ channels: The EAG family of voltage-gated K^+ channels comprises at least two members, $K_V10.1$ (EAG1) and $K_V11.1$ (hERG1), which are deeply involved in the regulation of different cancer hallmarks (Pardo and Stühmer, 2014). These channels have been identified as a potential target for anticancer therapies (Arcangeli and Becchetti, 2017; Xu et al., 2018), and both channels are expressed in PDAC ($K_V10.1$ (Gómez-Varela et al., 2007); $K_V11.1$ (Feng et al., 2014; Lastraioli et al., 2015)).

In the healthy organism, $K_V10.1$ and $K_V11.1$ are expressed in excitable cells such as neurons and muscle cells. In addition to its expression in PDAC, both channels have been detected in many other tumor cell lines and primary tumors including neuroblastoma (Meyer and Heinemann, 1998; Pardo et al., 1999), melanoma (Nilius and Wohlrab, 1992; Meyer et al., 1999; Gavrilova-Ruch et al., 2002) as well as different tumors of epithelial origin (Ouadid-Ahidouch et al., 2001; Lastraioli et al., 2004; Hemmerlein et al., 2006; Ding et al., 2007; Ousingsawat et al., 2007), and leukemias (Pillozzi et al., 2002). The expression of $K_V10.1$ seems to correlate with high-grade tumors and may confer a proliferative advantage for tumor cells (Comes et al., 2015).

$K_V11.1$ channels are preferentially expressed in cardiac myocytes and required for the ordered repolarization of cardiac action potentials. $K_V11.1$ expression in cancer cells has also been linked to high-grade tumors and has been strongly implicated in cell proliferation and migration of several cancers (Comes et al., 2015). $K_V11.1$ expression is elevated in PDAC tumor cells, in particular in lymph node–positive PDAC (Feng et al., 2014). In contrast, cells of the tumor stroma and the normal ductal epithelium do not express $K_V11.1$ (Lastraioli et al., 2015). $K_V11.1$ supports cancer cell proliferation, vitality, migration, and invasion also in several PDAC cell lines (J. Feng et al., 2014; E. Lastraioli et al., 2015; Zhi et al., 2017). It is involved in cell cycle regulation as $K_V11.1$ silencing promotes cell cycle arrest in the G_1 phase (Feng et al., 2014). In primary PDAC cultures, $K_V11.1$ blockage was found to be cytotoxic.

$K_V11.1$ physically and functionally interacts with other plasma membrane proteins, such as the epidermal growth factor receptor (EGF-R) and adhesion receptors of the integrin family (Arcangeli and Becchetti, 2006; Lastraioli et al., 2015), which strongly contribute to PDAC aggressiveness (Sun et al., 2018). In addition, EGF-R inhibition represents one of the therapeutic strategies for nonresectable PDAC (Hessmann et al., 2020). The interaction between $K_V11.1$ and EGF-R stimulates an EGF-R–dependent phosphorylation of ERK1 and ERK2, which are key signaling proteins downstream to EGF-R, and are involved in cell proliferation (Lastraioli et al., 2015). As stated in the introduction about K^+ channels, $K_V11.1$ modulates cell proliferation through a conductive mechanism by its impact on the cell membrane potential (Becchetti et al., 2017). $K_V11.1$ also regulates cell migratory programs of PDAC cells by modulating stress fiber dynamics and f-actin organization by its impact on the intracellular Ca^{2+} concentration (Manoli et al., 2019). This effect relies on nonconductive mechanisms and is based on the formation of a complex with β1 integrins, which leads to the activation of downstream signaling processes involving paxillin.

$K_V11.1$ is a target for both posttranscriptional and posttranslational modifications by small noncoding RNA molecules (miRNAs). miRNAs participate in human tumorigenesis and/or metastasis because of their ability to target oncogenes and/or tumor suppressors (Feng et al., 2014). $K_V11.1$ is a direct target of mir-96 and mir-493 in human PDAC (Feng et al., 2014; Zhi et al., 2017; Xu et al., 2018), where both miRNAs are down-regulated. These data are recapitulated in PDAC cell lines. *In vivo* and *in vitro*, mir-96 and mir-493 silencing increases proliferation, migration, and invasion of PDAC cells, while their overexpression highly suppresses tumorigenicity and metastasis of PDAC. These observations suggest that the above miRNAs can work as tumor suppressors in PDAC in a $K_V11.1$-dependent manner (Feng et al., 2014; Zhi et al., 2017).

$K_{Ca}3.1$ channels: $K_{Ca}3.1$ channels are functionally expressed in pancreatic ducts and are part of the transepithelial ion and fluid transport machinery (Hayashi et al., 2012; Wang et al., 2013). $K_{Ca}3.1$ channels are found in the luminal and basolateral membranes in the intercalated and interlobular ducts of the pancreas (Hayashi and Novak, 2013).

$K_{Ca}3.1$ is one of the first K^+ channels that were found to be massively overexpressed in primary pancreatic cancer samples and to be functional in several pancreatic cancer cell lines (Jäger et al., 2004). Such findings were later reproduced by other groups (Zaccagnino et al., 2016; Jiang et al., 2017, 2019). $K_{Ca}3.1$ expression rises in a stepwise fashion during the dedifferentitation process from the normal pancreas to PanINs and PDAC (Jiang et al., 2017). The clinical relevance of this finding is underscored by the correlation of increased $K_{Ca}3.1$ channel expression and patient prognosis: high $K_{Ca}3.1$ channel expression is associated with poor patient survival. The predictive power of $K_{Ca}3.1$ expression is not limited to PDAC. It also applies to several other cancer entities including, among others, breast (Faouzi et al., 2016), lung (Bulk et al., 2015), and ovarian cancer (Zhao et al., 2016).

Mechanistically, $K_{Ca}3.1$ channels regulate pancreatic cancer cell behavior in several ways. First of all, they provide the electrochemical driving force needed for Ca^{2+} entry by counterbalancing the depolarization of the membrane potential caused by Ca^{2+} influx channels such as TRP channels or Cl^- efflux through anion channels. The former has been observed in PSCs

(Storck et al., 2017) and is known from many immune cells such as macrophages (Gao et al., 2010) and lymphocytes (see *Ion Channel Involvement in Desmoplasia*). The latter appears to be relevant for pancreatic cancer cells. $K_{Ca}3.1$ channels interact with the gamma-aminobutyric acid (GABA) receptor subunit pi (GABRP). Thereby, they maintain the cell membrane potential and allow efficient Ca^{2+} signaling to enhance CXCL5-CCL20 secretion. This, in turn, causes macrophage infiltration into the cancer tissue and tumor growth (Jiang et al., 2019). $K_{Ca}3.1$-mediated K^+ efflux is also necessary for volume dynamics during the cell cycle (Bonito et al., 2016) and migration. Accordingly, $K_{Ca}3.1$ can promote tumor progression by modulating cell proliferation as well as cell migration and invasion (Schwab et al., 2012; Bonito et al., 2016). Finally, $K_{Ca}3.1$ channels are not only expressed in the plasma membrane but also in the inner membrane of mitochondria (De Marchi et al., 2009). There is indirect evidence that $K_{Ca}3.1$ channels are also present in the mitochondria of pancreatic cancer cells and regulate metabolic activity of mitochondria, potentially by modulating their membrane potential (Kovalenko et al., 2016). However, the relative importance of the plasma membrane vs. mitochondrial $K_{Ca}3.1$ channels in regulating the cellular metabolism still remains to be determined. The common link could be the intracellular Ca^{2+} concentration, which also affects mitochondrial function (Delierneux et al., 2020).

K_{2P} channels: There is very limited information about K_{2P} channels in pancreatic cancer. A systematic review of public databases identified the up- or down-regulation of $K_{2P}1.1$ or $K_{2P}3.1$ mRNA, respectively. However, these findings were not complemented by any functional data (Williams et al., 2013). $K_{2P}2.1$ modulates migration and proliferation of PDAC cell lines (Sauter et al., 2016).

TRP Channels in Pancreatic Cancer Cells

TRPM channels: An analysis of published genomic data from PDAC patients revealed an overexpression and the occurrence of somatic mutations of TRPM2. Both of them are negatively correlated with patient survival. TRPM2 overexpression or silencing modulates migration and proliferation of a PDAC cell line. So far, it remains to be determined how the somatic mutations of TRPM2 affect channel activity (Lin et al., 2018).

Similar observations were made for TRPM7. It is overexpressed in PDAC tissue, and this correlates with poor patient survival (Rybarczyk et al., 2012; Rybarczyk et al., 2017) as well as increased tumor size and advanced PDAC stages (Yee et al., 2015). In the zebra fish model, TRPM7 contributes to the development of the pancreas and carcinogenesis (Yee et al., 2011). Somatic TRPM7 mutations have been detected in several cancer entities (reviewed in Yee (2017)). Their functional significance has yet to be determined. On the cellular level, TRPM7 regulates proliferation and cell cycle progression (Yee et al., 2011). In zebra fish, the defects in cell cycle progression of the *trpm7*^{b508} mutants can be partially rescued by supplementary Mg^{2+} (Yee et al., 2011). TRPM7 knockdown reduces PDAC cell chemotaxis and invasion (Yee et al., 2015), at least in part by regulating the intracellular Mg^{2+} homeostasis and via the Hsp90α/uPA/MMP-2 proteolytic axis (Rybarczyk et al., 2012, Rybarczyk et al., 2017).

TRPM8 is also overexpressed in human PDAC compared to normal tissue and required for cell proliferation (Yee et al., 2010). PDAC cells express functional TRPM8 channels as shown by whole-cell patch-clamp experiments. Channel activation inhibits PDAC cell motility (Cucu et al., 2014). Moreover, TRPM8 silencing increases the sensitivity to gemcitabine (Liu et al., 2018).

TRPV6: The high Ca^{2+} selectivity is a distinguishing feature of TRPV6 (and TRPV5) channels (Fecher-Trost et al., 2017). So far, there are only very few publications on TRPV6 channels in pancreas physiology and pathophysiology. Immunohistochemistry revealed their expression in acinar cells (Zhuang et al., 2002). A transcriptomic analysis indicates however that they are expressed at higher levels in the ductal epithelium (Segerstolpe et al., 2016).

Overexpression of TRPV6 appears to be common in cancers of epithelial origin. Thus, its tumor-promoting role in prostate cancer is well established (Raphaël et al., 2014). However, there is a controversy with respect to TRPV6 expression in PDAC. While Song et al. (2018) reported an overexpression, we found a reduced expression in microdissected PDAC samples (Zaccagnino et al., 2016). However, both of these studies did not take into account whether the tissue samples were from invasive or noninvasive parts of the tumor. This is a relevant distinction: A preponderance of TRPV6 expression was shown for the invasive parts of breast cancer (Dhennin-Duthille et al., 2011). Loss-of-function variants of TRPV6 channels are linked to another pancreas pathology: Early onset chronic pancreatitis (Masamune et al., 2020). We already mentioned in the introduction that an early onset (hereditary) chronic pancreatitis, which can also be caused by a mutation of the CFTR channel, leads to an increased risk to develop PDAC (Becker et al., 2014). The potential clinical relevance of TRPV6 channels in PDAC is further underpinned by observations from a phase I dose escalation study with the TRPV6 inhibitor SOR-C13 in cancer patients. Stable disease and a reduction in the CA 19-9 tumor biomarker were observed in both PDAC patients participating in this study (Fu et al., 2017).

Cl^- Channels in Pancreatic Cancer Cells

ANO1 (TMEM16A) is a Ca^{2+}-activated Cl^- channel (CaCC). In freshly isolated murine pancreatic acini, HCO_3^- exits the cells through the apical ANO1 channel, which controls luminal pH balance. Luminal pH may be perturbed by the exocytotic release of the acid content of zymogen granules, both under physiologic condition and upon supramaximal stimulation, which represents an *in vitro* model of acute pancreatitis (Han et al., 2016). In acute pancreatitis, IL-6 promotes ANO1 expression via IL-6R/STAT3 signaling. ANO1 overexpression, in turn, increases IL-6 secretion via IP_3R/Ca^{2+}/NFκB signaling activation (Wang et al., 2020a). Thus, ANO1 appears to be involved in a positive feedback loop in this inflammatory disorder.

CFTR and ANO1 are highly expressed in Capan-1 cells, where they mediate ATP/UTP-regulated Cl^- secretion (Wang et al., 2013). ANO1 is overexpressed in several PDAC cell lines when its expression is compared to that in HDPE cells which are suggested

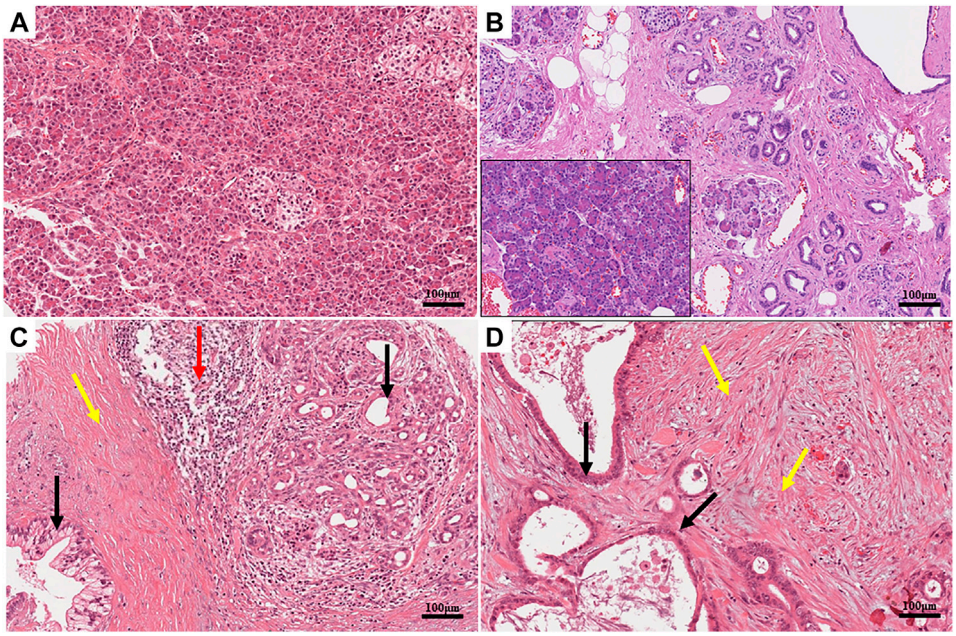


FIGURE 1 | (A) Histomorphology of a healthy human pancreas, hematoxylin and eosin (H&E). The parenchymal structure of the organ is clearly visible. Acinar cells are identifiable by their typical round shape. Their bases are stained in blue due to the presence of the nuclei, while their apices are pink due to the high concentration of zymogen. Two islets are located in the central and right parts of the image. The cytoplasm of the islet cells is paler than the surrounding acinar cells. **(B)** Histomorphology of a chronic pancreatitis, hematoxylin and eosin (H&E). The tissue is characterized by an evident increase in interlobular fibrosis, atrophy of the acini, and inflammatory infiltrate, which is evident when compared to the healthy component of the same sample (inset). **(C,D)** Histomorphology of two human pancreatic ductal adenocarcinomas (PDACs), hematoxylin and eosin (H&E). The normal architecture of the parenchyma is lost. Multiple layers of cells highlight the neoplastic lesions in panel **(C)**. High levels of desmoplasia (colored in pink) are present especially in panel **(D)**. Distribution of different cell populations is detectable in the tumor tissue; neoplastic cells (pointed by black arrows) are embedded in a dense desmoplastic stroma (pointed by yellow arrows). Evident immune cells infiltration (pointed by red arrows) is present on the right side of the figure. Immune cells are identifiable by their small sizes and the intense basophilic staining of the nuclei. Scale bars: 100 µm.

to represent a model of the normal human pancreatic ductal epithelium (Sauter et al., 2015). Indeed, the analysis of patient material shows that ANO1 mRNA and protein are up-regulated in 75% of the cases. This is associated with a poor probability of survival (Crottès et al., 2019).

An EGFR-related signaling pathway, requiring ANO1, regulates Cl^- and Ca^{2+} homeostasis in pancreatic cancer cells. This EGF-induced store-operated Ca^{2+} entry is required for the migration of pancreatic cancer cells (Crottès et al., 2019). Interestingly, ANO1 has a promigratory role in PDAC cells but has no effect on cell proliferation. Whole-cell patch-clamp experiments reveal functional ANO1 as a major mediator of PDAC CaCC currents. While knockdown of ANO1 using siRNA nearly completely abolishes the CaCC-mediated currents, the three tested ANO1 inhibitors T16Ainh-A01, CaCCinh-A01, and NS3728 show unspecific side effects and limited specificity (Sauter et al., 2015).

ION CHANNEL INVOLVEMENT IN DESMOPLASIA

Fibrosis is a pathological outcome common for many chronic inflammatory diseases including chronic pancreatitis (Wynn and Ramalingam, 2012). The abundant stroma reaction (desmoplasia) is a hallmark common to both chronic pancreatitis and PDAC (Haeberle et al., 2018). Chronic pancreatitis is considered a risk factor for pancreatic cancer, and indeed, it frequently evolves to a true PDAC (McKay et al., 2008). In both cases, the normal pancreatic parenchyma is markedly remodeled (as shown in **Figure 1**) so that the normal organ function is eventually lost. The poorly vascularized desmoplastic tissue is characterized by high stiffness, low elasticity, and high tissue pressure (up to 100 mmHg) (Stylianopoulos et al., 2012; Fels et al., 2016; Pethő et al., 2019), which leads to impaired perfusion of the tumor tissue with the further result of tissue hypoxia. The pancreatic stellate cells (PSCs) are believed to be the key effectors behind the stroma deposition in PDAC and chronic pancreatitis (Haeberle et al., 2018). Desmoplasia represents an important challenge that new PDAC therapies have to deal with (Henke et al., 2020). The absence of vascularization combined with vessel compression because of the massive fibrosis prevents the efficient delivery of the chemotherapeutic drugs (Dauer et al., 2018).

Consequently, new strategies targeting the stroma compartment have emerged. This includes the attempt to attenuate/reverse the activation of the cancer-associated fibroblasts (CAFs) which also includes PSCs. The results of these studies however are contradictory. Inhibiting the TGF-β signaling pathway with the anticancer compound Minnelide,

which is able to reverse the activation state of the CAFs, has a similar positive effect in a murine PDAC model (Dauer et al., 2018) as the inhibition of hedgehog signaling in CAFs with IPI-926. Moreover, IPI-926 also increases the delivery and the efficacy of gemcitabine in mice (Olive et al., 2011). However, other studies highlighted that an uncontrolled depletion of the stroma compartment rather promotes PDAC progression than slowing it down (Özdemir et al., 2014). Consequently, the understanding of the stromal compartment in PDAC has to be further refined. It has become apparent that cancer-associated fibroblasts constitute a heterogeneous cell population with distinct gene expression profiles, location within the tumor, and function (Von Ahrens et al., 2017; Öhlund et al., 2017; Miyai et al., 2020). Öhlund et al. propose a distinction between inflammatory fibroblasts, mainly responsible for the secretion of inflammatory factors, and myofibroblasts that are responsible for the ECM production (Öhlund et al., 2017). PSCs are included in this last category. To the best of our knowledge, it has not yet been studied whether these two types of CAFs are also equipped with distinct sets of ion channels.

Pancreatic Stellate Cells

In a healthy pancreas, PSCs are usually kept in a quiescent state, and they are responsible for the maintenance of the tissue integrity by regulating the ECM turnover (Haeberle et al., 2018). In PDAC, PSCs become strongly activated by the secretome and the physicochemical properties of the PDAC microenvironment (Omary et al., 2007a). Thus, PSCs are activated among others by inflammatory mediators, growth factors (PDGF and TGF-β1), cytokines (IL-1, IL-6, and IL-8), hormones, angiotensin II, intracellular signaling molecules, and reactive oxygen species (ROS) as well as hypoxia (Nielsen et al., 2017) and mechanical stimuli (Omary et al., 2007b; Fels et al., 2016, Fels et al., 2018; Ferdek and Jakubowska, 2017; Lachowski et al., 2017). Activated PSCs, in turn, secrete growth factors themselves so that they are engaged in a mutual positive feedback loop of other cells of the PDAC tissue (Fu et al., 2018). In addition, activated PSCs proliferate, migrate (Omary et al., 2007b), and secrete copious amounts of ECM components, especially collagen I and III (Ferdek and Jakubowska, 2017). The resulting changes in the pH values and increased stiffness of the desmoplastic tissue also feed back onto the behavior of PSCs (Lachowski et al., 2017). One of the mechanosensitive ion channels, Piezo1, that senses the mechanical properties of the PDAC microenvironment is inhibited by an acidic pH. This could prevent PSCs to be overridden by the mechanically triggered Ca^{2+} influx via Piezo1 channels (Kuntze et al., 2020).

Ion Channels and Fibrosis

The function of ion channels in tumor stroma cells is far from being fully understood, especially regarding PDAC. Nonetheless, we already know that some ion channels play a significant role in the development of fibrosis in other organs such as $K_{Ca}3.1$ in lungs, kidneys (Roach et al., 2013), and heart (Zhao et al., 2015); $K_{2P}2.1$ in cardiac fibrosis (Abraham et al., 2018); and TRPV4 in liver (Songa et al., 2014), heart (Adapala et al., 2013), and lung fibrosis (Rahaman et al., 2014). Usually the inhibition of these ion channels attenuates the profibrotic response of the fibroblasts (Cruse et al., 2011; Adapala et al., 2013; Rahaman et al., 2014; Abraham et al., 2018; Roach and Bradding, 2019).

Ion channel research in PSCs is still in its infancy. We will therefore draw some analogies from hepatic stellate cells that are closely related to PSCs and in which these ion channels may play a similar role. Hepatic stellate cells are responsible for matrix homeostasis in healthy livers (Puche et al., 2013). Similar to the PSCs in PDAC, they are mainly responsible for the excessive production and remodeling of the ECM in the fibrotic liver (Puche et al., 2013; Freise et al., 2015; Ezhilarasan et al., 2018). For this reason, these types of cells have been suggested as a possible target for antifibrotic therapy.

$K_{Ca}3.1$: We do not have much information on the role of $K_{Ca}3.1$ channels in PDAC-associated fibrosis, which is largely driven by PSCs. It is only known that $K_{Ca}3.1$ channels regulate migration of PSCs (Storck et al., 2017).

So far, it is under debate whether $K_{Ca}3.1$ has pro- or antifibrotic effects in the liver (Roach and Bradding, 2019). $K_{Ca}3.1$ expression is increased in hepatic stellate cells after the incubation with TGF-β, a known activator of hepatic stellate cells. In both *in vitro* and *in vivo* experiments, the inhibition of $K_{Ca}3.1$ shows an antifibrotic effect and decreases the expression of profibrotic genes (Freise et al., 2015). On the contrary, in the work of Møller et al., the inhibition or the absence of $K_{Ca}3.1$ in hepatic stellate cells and hepatocytes worsens liver fibrosis (Møller et al., 2016). This information highlights the possible problems that ion channel therapies could face; the inhibition of an ion channel expressed in different cell types could have different effects.

$K_{2P}2.1$: So far, we only know that PSCs express $K_{2P}2.1$ (previously designated as TWIK-related potassium channel-1; TREK1) (Fels et al., 2016). In fact, $K_{2P}2.1$ is a mechanosensitive ion channel that can be modulated by pressure and membrane stretch (Lauritzen et al., 2005; Honoré, 2007) but also by pH. $K_{2P}2.1$ contributes to setting the resting membrane potential of the cells (Bittner et al., 2014), and it is strongly correlated with proliferation and cell cycle in some tumors (Pethő et al., 2019). The mechanosensitive function of $K_{2P}2.1$ is postulated to be involved in the migration, especially in the coordination of the front and rear ends of the cells (Pethő et al., 2019). Sauter et al. observed that the activation of $K_{2P}2.1$ with BL 1249 in a PDAC line, BxPC-3, inhibits cell proliferation and migration through the hyperpolarization of the membrane (Sauter et al., 2016). Controversially, the absence of $K_{2P}2.1$ in heart myofibroblasts from pressure-overloaded mice attenuates cardiac fibrosis also by decreasing fibroblast proliferation and migration (Abraham et al., 2018). This highlights again how the same ion channel can have a different impact on the behavior of different cell types and how this topic must be considered during the development of new therapies. However, it may also be seen as an indication that the "natural," possibly fluctuating, activity is what matters physiologically. Clamping channel activity to a maximum or a minimum impairs cell function. It remains to be seen whether

TABLE 2 | Ion channel expression and function in innate and adaptive immune cells of pancreatic ductal adenocarcinoma.

Channel	Function	Reference
Neutrophils		
$K_{Ca}3.1$	Chemotaxis	Henríquez et al. (2016)
$K_{ir}2.1$	Possible role in neutrophil proliferation, membrane potential regulation, and Ca^{2+} influx	Masia et al. (2015)
$K_V1.3$	Membrane potential regulation and electric field detection	Kindzelskii and Petty (2005)
TRPC1	fMLF-stimulated migration and chemotaxis	Lindemann et al. (2015)
TRPC6	Chemotaxis and CXCL1-induced recruitment from the vasculature	Lindemann et al. (2013) and Lindemann et al. (2020)
TRPM2	*In vitro* transmigration	Yamamoto et al. (2008)
P2X7	IL-1β secretion	Karmakar et al. (2016)
H_V1	Ca^{2+} entry regulation, ROS production, and neutrophil migration	El Chemaly et al. (2010) Ramsey et al. (2009)
Monocytes/macrophages		
$K_{Ca}3.1$	M1 polarization	Xu et al. (2017)
$K_{2P}6.1$	Inflammasome formation	Di et al. (2018)
TRPC1	M1 polarization	Chauhan et al. (2018)
TRPM2	Chemokine production	Yamamoto et al. (2008)
TRPM7	Ca^{2+}-induced macrophage stimulation, proliferation, and M2 polarization	Schilling et al. (2014) and Schappe et al. (2018)
H_V1	Phagosomal pH regulation and ROS production	El Chemaly et al. (2014)
Dendritic cells		
$K_V1.3$, $K_V1.5$	MHCII expression, migration, and cytokine production	Matzner et al. (2008)
$Na_V1.7$	Migration	Zsiros et al. (2009)
P2X7	Antigen presentation and migration	Mutini et al. (1999) and Saéz et al. (2017)
H_V1	ROS production	Szteyn et al. (2012)
Myeloid-derived suppressor cells (MDSCs)		
TRPV1	Promotes MDSC formation	Hegde et al. (2011)
P2X7	ARG-1, TGF- β1, and ROS up-regulation	Bianchi et al. (2014)
NK cells		
$K_{Ca}3.1$	Negatively influencing proliferation, degranulation, and cytotoxicity	Koshy et al. (2013)
$K_V1.3$	Positively influencing proliferation and degranulation	Koshy et al. (2013)
$CD4^+$ and $CD8^+$ T-cells		
$K_{Ca}3.1$	Sustaining Ca^{2+} influx during T-cell activation	Ghanshani et al. (2000) and Wulff et al. (2003)
$K_V1.3$	Sustaining Ca^{2+} influx during T-cell activation	Wulff et al. (2003)
TRPM4	Motility and cytokine production	Weber et al. (2010)
CRAC[a]	Ca^{2+} influx during T-cell activation	Feske et al. (2012)
T_{reg}s		
$K_{Ca}3.1$	Still unclear	Estes et al. (2008)
$K_V1.3$	Still unclear	Varga et al. (2009)
CRAC[b]	Development and differentiation	Vaeth et al. (2019)
B cells		
$K_{Ca}3.1$	Sustaining Ca^{2+} influx during B-cell activation	Wulff et al. (2004)
$K_V1.3$	Sustaining Ca^{2+} influx during B-cell activation	Wulff et al. (2004)
CRAC[c]	Ca^{2+} influx during B-cell activation	Feske et al. (2012)

[a]*Murine T-cells: mRNA and fluorescence-based data indicate that T-cells up-regulate* Orai1 *and down-regulate* Orai2 *when they become activated (Vaeth et al., 2017). The role of* Orai3 *is controversial (McCarl et al., 2010; Vaeth et al., 2017).*

Human peripheral T-cells: the dominant isoform is Orai1*, but all the three genes are up-regulated upon activation (Lioudyno et al., 2008). There is no difference in cell surface expression of ORAI1 between human memory and naive T-cells (Cox et al., 2013).*

[b]*Murine peripheral Tregs: mRNA data suggest the expression of* Orai1 *and* Orai2*, while much less of* Orai3 *(Vaeth et al., 2017).*

Human peripheral T_{reg}s: ORAI1 and ORAI2, but not ORAI3, were detected using immunocytofluorescence. The expression of Orai1 *in T_{reg}s is significantly inferior compared to naive and activated $CD4^+$ T-cells (Jin et al., 2013).*

[c]*Murine B cells express* Orai1*,* Orai2 *and* Orai3 *to a comparable extent (Gwack et al., 2008; Vaeth et al., 2017).*

Human B cells: no detailed mRNA data. There is no difference in cell surface expression of ORAI1 between memory and naive B cells (Cox et al., 2013).

$K_{2P}2.1$ channels exert a similar role in PDAC desmoplasia, where the unique tumor microenvironment could influence $K_{2P}2.1$ function in many ways.

TRPV4: The transient receptor potential vanilloid channel 4 (TRPV4) is a mechanosensitive Ca^{2+}-permeable nonselective cation channel that is expressed in many organs including the pancreas (Zhan and Li, 2018). TRPV4 is also expressed in PSCs. Its mRNA expression strongly decreases in PSCs when they are cultured under an elevated ambient pressure (+100 mmHg), mimicking the conditions that can be found in PDAC (Fels et al., 2016; Pethő et al., 2019; Sharma et al., 2019). The functional implications of this mechanosensitive expression have not yet been published. The decreased TRPV4 mRNA expression upon mechanical stimulation can be explained as a compensatory response of the cells which prevents Ca^{2+} overload following the pressure stimulus (Fels et al., 2016).

Notably, PSCs also release TGF-β upon stimulation with pressure (Sakata et al., 2004; Fels et al., 2016). TRPV4 integrates mechanical stimuli and soluble signals such as TGF-β, and it drives the epithelial–mesenchymal transition (EMT) (Adapala et al., 2013; Sharma et al., 2019). TRPV4 expression is dramatically increased in many tissue samples of patients with

liver fibrosis (Songa et al., 2014). Furthermore, TRPV4 is highly expressed in hepatic stellate cells (Songa et al., 2014). Inhibition of TRPV4 decreases cell proliferation of hepatic stellate cells, decreases their TGF-β–dependent activation and the expression of collagen α1 and α-smooth muscle actin genes in *in vitro* cultures (Songa et al., 2014). Inhibition of TRPV4 also leads to an increase in apoptosis and inhibition of autophagy in the TGF-β–treated hepatic stellate cell line HCS-T6. These findings can be taken as indication for a similar role of TRPV4 channels in PSCs as well.

IMMUNITY AND PDAC

The description/staging of cancers has significantly evolved over the last decades to include the tumor microenvironment (TME) and the infiltration of the tumors by the immune system (e.g., Immunoscore® for colorectal cancers (Galon et al., 2014)). This is particularly important since T-cell infiltration, in general, bears a good prognostic feature: high $CD4^+$ and $CD8^+$ densities are associated with better overall and disease-free survival (Tang et al., 2014; Knudsen et al., 2017; Lohneis et al., 2017; Nejati et al., 2017).

The development of PDAC can be seen as a result of failed removal of malignant cells (Dunn et al., 2002). This failure might originate from the quantitative and qualitative composition of the immune cell repertoire in the TME, and/or altered function of the immune cells and their ion channels. An in-depth analysis of the immune cells in PDAC is beyond the scope of this review, and thus, we will use a simplified classification scheme and focus on the roles of the cells of innate and adaptive immunity in PDAC progression and how their roles may be modulated by ion channels. The expression of ion channels in immune cells in PDAC is summarized in **Table 2**. Addition of the immune component to a topical review on ion channels in PDAC is unique to this article, and thus, basic functions of immune cells have to be discussed briefly in the corresponding sections about a cell type.

Based on the relative proportion of $CD3^+$ and $CD8^+$ cells over all cells in the tumor (Galon and Bruni, 2019), PDAC is often ranked among the "coldest" human tumors (Maleki Vareki, 2018). Although leukocytes ($CD45^+$ cells) comprise almost 50% of all cells isolated from murine (Clark et al., 2007) and human PDAC (Trovato et al., 2019), T lymphocytes are significantly less abundant (15% of total cells in mice and ca. 20% in humans) compared to well-known "hot" tumors like melanoma (Sakellariou-Thompson et al., 2017; Blando et al., 2019). Low T-cell infiltration of PDAC can be due to a desmoplastic mechanical impediment, hypoxia, and low extracellular pH (Knudsen et al., 2017).

At the time of diagnosis, the TME is already highly immunosuppressive, which can be related to the high number of myeloid-derived suppressor cells in PDAC (Trovato et al., 2019). Moreover, the low pH and the alterations of the ionic composition of the TME may lead to the formation of tumor-associated immune cells which become the malfunctioning side of the immune response (Vesely et al., 2011; Gabrilovich et al., 2012; Girault et al., 2020). The fact that ion channels are expressed in both antitumor and protumor/suppressor immune cells allows us to consider ion channels as putative mediators of the biased immune response in PDAC (Feske et al., 2015; Fels et al., 2018).

Ionic Composition of the Tumor Microenvironment

Distinct characteristics of PDAC, that is, poor vascularization and a markedly fibrotic stroma, result in deficient oxygen supply and metabolite accumulation (Olive et al., 2009; Provenzano et al., 2012). The high metabolic rate, glycolysis (GAPDH activity; production of lactate (Dovmark et al., 2017)), implementation of the pentose phosphate pathway, and production of CO_2 are the source of protons which lead to extracellular acidification in PDAC (Gillies et al., 2002; Hashim et al., 2011; High et al., 2019). Such an acidification of poorly perfused tumor areas has a profound impact on the function of ion channels in all cells of the tumor tissue (reviewed in Pethő et al. (2020)). Acidification, severe hypoxia, and mechanical stress also cause cell necrosis. This is associated with an elevation of the $[K^+]$ in the interstitium (Cruz-Monserrate et al., 2014; Eil et al., 2016; Leslie et al., 2019). Moreover, the concentration of Na^+, a major contributor of osmotic pressure in the interstitium, is increased, which can have multiple implications for the infiltration of immune cells (He et al., 2020). Thus, the ionic composition of the tumor microenvironment is characterized by altered concentration gradients across the plasma membrane, that is, by altered electrochemical driving forces and by constituents, for example, protons, which have a strong impact on channel activities. Importantly, the disrupted ionic composition is sensed by ion channels in cancer, immune, and stromal cells and inevitably affects their function. The consequences of the altered tumor environment on cell function through the modification of ion channels of immune cells will be discussed in the following.

Cells of the Innate Immune Response

Neutrophils: A high number of neutrophils in the PDAC stroma is usually associated with poor prognosis (Wang et al., 2018; Oberg et al., 2019). Likewise, a high neutrophil-to-lymphocyte ratio (NLR), also in peripheral blood, is associated with a lower 5-year survival rate after tumor resection (Nywening et al., 2018).

Expression of voltage-gated and Ca^{2+}-activated channels, $K_V1.3$ and $K_{Ca}3.1$, was shown in murine and human neutrophils (Krause and Welsh, 1990; Kindzelskii and Petty, 2005; Henríquez et al., 2016). Moreover, murine neutrophils express electrophysiologically detected inwardly rectifying $K_{ir}2.1$ channels which are also assumed to contribute to their resting membrane potential and Ca^{2+} influx (Masia et al., 2015).

Since K^+ channels are involved in neutrophil migration and chemotaxis like other cells present in the PDAC microenvironment, a high extracellular K^+ concentration may also perturb neutrophil function. This assumption is important not only in the context of their ability to reach the cancer niche but may also be a cause of unfavorable retention of neutrophils in PDAC milieu.

While *intracellular* ATP is a regulator of neutrophils' Kir6.x channels (Silva-Santos et al., 2002), *extracellular* ATP, for example, released from necrotic cells, induces neutrophil recruitment through purinergic P2X7 receptor activation (McDonald et al., 2010). Opening of the ATP-gated P2X channels leads to Ca^{2+}/Na^{+} influx (Karmakar et al., 2016). Importantly, P2X7 is expressed also in cancer and PSCs, and the P2X7 inhibitor, AZ10606120, reduces cancer cell proliferation *in vitro* and *in vivo* (Haanes et al., 2012; Giannuzzo et al., 2015).

Ca^{2+} signaling plays a major role in neutrophil migration, phagocytosis, and ROS production. One of the key mechanisms in the Ca^{2+} increase is mediated by store-operated Ca^{2+} entry (SOCE) and subsequent activation of Orai1 channels. Several other Ca^{2+}-permeable TRP channels are also involved in the innate immune response (Najder et al., 2018). Since neutrophils express C-X-C chemokine receptor–type 2 (CXCR2), they are attracted by ligands like CXCL1/IL-8, CXCL2, and CXCL5, released in pancreatitis and pancreatic cancer (Saurer et al., 2000; Steele et al., 2016; Najder et al., 2018; Wu et al., 2019; Zhang et al., 2020). Indeed, inhibition of CXCR2 signaling in PDAC shows beneficial results (Ijichi et al., 2011; Steele et al., 2016). Recruitment of neutrophils upon CXCR2 activation is mediated by Na^{+} and Ca^{2+}-permeable, classical/canonical transient receptor potential 6 (TRPC6) channel (Lindemann et al., 2013, 2020). TRPC6 is also expressed in PSCs, where it mediates hypoxia-induced migration and production of cytokines (Nielsen et al., 2017). In a mouse model, it could be shown that inhibition of TRPC6 with specific antagonists (SAR7334, BI-749327) diminishes the inflammatory response in the lungs and ameliorates cardiac and renal fibrosis (Lin et al., 2019; Chen et al., 2020). One can presume that such a beneficial effect could also be elicited in PDAC, in part by inhibiting neutrophil recruitment into the tumor.

In colorectal cancer, another chemokine receptor, formyl peptide receptor (FPR1), is highly expressed in tumor-infiltrating, myeloperoxidase-positive (MPO^{+}) cells (Li et al., 2017). Also, FPR1 is enriched in immune cells of the recently suggested L4 PDAC subtype (Zhao et al., 2018). In murine neutrophils, FPR1-mediated directed migration depends on TRPC1 channels which may therefore contribute to neutrophil infiltration in PDAC (Lindemann et al., 2015; Fels et al., 2018).

Once at the target, activated neutrophils produce ROS, release metalloproteinases (e.g., MMP-9) and cytokines, and form neutrophil extracellular traps (NETs) (Wu et al., 2019). The remarkable ability of neutrophils to produce ROS depends on the depolarizing activity of the NADPH oxidase (NOX2) and concomitant action of voltage-gated proton channels (H_V1) (DeCoursey et al., 2016). Their activity in neutrophils is very relevant for PDAC progression. Neutrophil-derived ROS may cause cancer apoptosis due to TRPM2 channel activation (Gershkovitz et al., 2018). Accordingly, pharmacological stimulation of ROS production induces pancreatic tumor cell apoptosis (Shi et al., 2008). However, channel expression in cancer cells can also promote cancer cell proliferation (Lin et al., 2018). Release of NETs, a defense mechanism of extruding DNA covered with enzymes and histones, is often ROS-dependent and is therefore indirectly mediated by H_V1 activity. NET formation can occlude pancreatic ducts, cause pancreatitis, and promote PDAC metastasis to the liver (Leppkes et al., 2016; Takesue et al., 2020). Also, distant PDAC metastasis is facilitated by activated neutrophils in the circulation (Tao et al., 2016). Thus, aiming at the H_V1 channel in cancer therapy could have potential benefits, mostly due to inhibition of ROS-related activity of neutrophils (Fernández et al., 2016).

Macrophages: Tumor-associated macrophages (TAMs) are generally divided into "classically activated" M1 and "alternatively activated" immunosuppressive M2 macrophages. The latter type is predominant in PDAC tissue (Habtezion et al., 2016; Hu et al., 2016; Liu et al., 2016). M2 polarization is induced by IL-4 and IL-13 (Biswas and Mantovani, 2010). The presence of these cells in PDAC is associated with poor prognosis. TAMs also contribute to formation of desmoplasia through interplay with PSCs and mutual stimulation of cytokine production. Moreover, macrophage-derived metalloproteinases mediate dynamic turnover of fibrotic tissue and allow for tumor expansion (reviewed in Hu et al. (2015)). Chemotherapy can induce macrophage polarization into the tumoricidal M1 type and improve therapy outcome (Kurahara et al., 2011; Di Caro et al., 2015). Macrophage recruitment to the tumor site is mediated by CCL2/CCR2 and CSF-1/CSF-1R axes, with the latter additionally promoting M2 polarization. Inhibition of these signaling pathways shows potential benefits so that the CCL2/CCR2 inhibitor (PF-04136309) is implemented in PDAC clinical trials (NCT01413022) (Zhu et al., 2014; Habtezion et al., 2016; Nywening et al., 2018).

Channels expressed in macrophages often overlap with those expressed in neutrophils, especially in regard to K^{+} channels (reviewed in: Feske et al. (2015)). There is also evidence that $K_{2P}6.1$ (TWIK2) mediates K^{+} efflux. In murine macrophages, $K_{2P}6.1$ leads to inflammasome formation and—in cooperation with the depolarizing action of P2X7—induces release of IL-1β (Di et al., 2018).

Ca^{2+} signaling plays a crucial role for macrophage function. Thus, migration and phagocytosis of macrophages depend on Ca^{2+}-permeable channels (Desai and Leitinger, 2014). In addition, the polarization of macrophages is mediated by several Ca^{2+}-permeable ion channels including few members of the TRP channel family. TRPM7 promotes M2 polarization and shows high activity in this type of macrophage (Schilling et al., 2014). In contrast, deletion of TRPM2 favors a proinflammatory macrophage phenotype in *Helicobacter pylori* infection (Beceiro et al., 2017). M1 macrophage polarization is promoted by the activity of TRPC1 and $K_{Ca}3.1$ (Xu et al., 2017; Chauhan et al., 2018). These channels could be taken under consideration in approaching macrophage plasticity in PDAC, since M2 macrophages comprise the majority of infiltrated immune cells. However, since TRPC1 and $K_{Ca}3.1$ channels are expressed not only in neutrophils but also in cancer and stromal cells as well as in lymphocytes, the impact of activating these channels is difficult to predict (see below, *Pharmacological Targeting of Ion Channels in PDAC* for a more detailed discussion).

Dendritic cells: There are a few dendritic cells at the tumor site and in the circulation of PDAC patients. The ability of dendritic cells to present foreign antigens has been used for designing dendritic cell–based immunotherapy (dendritic cell vaccines) against pancreatic cancer (Deicher et al., 2018). Some data indicate that dendritic cells predominantly support immunological tolerance in the strongly immunosuppressive PDAC environment (Barilla et al., 2019). Encountering an antigen elicits $[Ca^{2+}]_i$ to rise in dendritic cells, which is mediated by CRAC channels. The voltage-gated K^+ channels, $K_V1.3$ and $K_V1.5$, modulate Ca^{2+} fluxes by hyperpolarizing the membrane potential. They are involved in major histocompatibility complex II expression, migration, cytokine production, and phagocytosis (Matzner et al., 2008). P2X7 is also expressed in murine dendritic cells, mediating antigen presentation and migration (Mutini et al., 1999; Saéz et al., 2017). However, despite their crucial role in coordinating the immune response, the involvement of ion channels in functions of dendritic cells present in PDAC tissue is not yet well-described.

Myeloid-derived suppressor cells: Myeloid-derived suppressor cells (MDSCs) are not fully differentiated myeloid cells which exhibit highly immunosuppressive features. They can be further divided into polymorphonuclear (PMN-MDSC) and monocytic (M-MDSC) in mice, and early-stage (eMDSC) MDSCs in human. They share some phenotypic features with differentiated myeloid cells but can be distinguished by their inhibitory properties (Trovato et al., 2019). PSCs are presumed culprits of promoting MDSCs in pancreatic cancer *via* IL-6 release (Mace et al., 2013). MDSCs themselves exhibit increased arginase 1 activity, depleting the tumor microenvironment of L-arginine, which, in turn, elicits T-cell suppression. Not surprisingly, MDSC depletion is a looked for method for PDAC treatment (Thyagarajan et al., 2019).

There is an immense lack of knowledge about the function of ion channels in MDSCs. P2X7 and TRPV1 are the only channels described in MDSCs so far. P2X7 activation in M-MDSCs increases arginase-1, TGF-β, and production of ROS (Bianchi et al., 2014). In mice, TRPV1 activation stimulates MDSCs and protects from hepatitis (Hegde et al., 2011). The role of MDSC ion channels in the PDAC environment still needs to be elucidated.

Natural killer cells: Natural killer cells (NK cells) are innate lymphoid cells, and their function is similar to that of cytotoxic $CD8^+$ cells. Despite the fact that their percentage ranges around 1.5–2% of mononuclear cells (Marsh et al., 2014; Bazhin et al., 2016) (which becomes 5% of leukocytes after a partial resection (Gürlevik et al., 2016)), their role is important. The intravenous injection of an NK cell line (LNK) into the tumor improves the survival of mice and delays PDAC growth (Hu et al., 2019b). Several clinical trials built on NK cell–based immunotherapy are on at the moment (Sunami and Kleeff, 2019).

The role of NK cells against cancer is now well documented, and their ion channels appear to be of pivotal importance (Redmond and Buchanan, 2017). Like essentially all other lymphoid cells, NK cells have CRAC currents and K^+ currents mediated by $K_V1.3$ and $K_{Ca}3.1$, which are crucial for their function (Redmond and Buchanan, 2017). Koshy et al. discovered that a minority of human NK cells, defined as adherent NK cells, is able to nearly double the number of their $K_V1.3$ and $K_{Ca}3.1$ channels after their activation by cocultured cancer cells ($K_V1.3$: 50 to 125/cell; $K_{Ca}3.1$: 20 to 40/cell). Contrariwise, the majority of NK cells, named "nonadherent," up-regulate only $K_V1.3$, while $K_{Ca}3.1$ channels remain unaltered ($K_V1.3$: 20 to 350/cell; $K_{Ca}3.1$: 20 to 15/cell) (Koshy et al., 2013).

Cells of the Adaptive Immune Response

The PDAC tissue is heavily infiltrated by different subsets of T-cells and B cells. Depending on the nature of the cells and the cytokines being secreted, these cells can be both protumoral and antitumoral. Unfortunately, very little information is available about the ion channel expression of the different T-cell subsets in PDAC. To set the frame for future research on ion channels in PDAC-associated T- and B-cell subsets, we will first summarize the general scheme about the dependence of T-cell activation on ion channels and then focus on the T- and B-cell subsets relevant in PDAC (**Figure 2A**) along with mostly non-PDAC–specific information available about the ion channel expression of those T- and B-cell subsets. **Figure 2B** provides an overview with respect to the expression (changes) in two of the most important K^+ channels found in the various subtypes.

Adaptive Immunity: Antitumor Cells

Principal ion channels in T-cells. The function of ion channels in T lymphocytes has been thoroughly investigated in the last three decades (Cahalan and Chandy, 2009). T lymphocyte activation strictly depends on extracellular Ca^{2+} entry via Ca^{2+} release–activated channels, CRAC, composed of Orai (three homologues: *Orai1*, *Orai2*, and *Orai3*) and Stim (two homologues: *Stim1* and *Stim2*) proteins (Feske et al., 2012). In human, T lymphocyte Orai1 is essential for correct T-cell functioning (Vaeth et al., 2017). Ca^{2+} entry is facilitated by the opening of two K^+ channels, the voltage-gated gated $K_V1.3$ and the Ca^{2+}-activated $K_{Ca}3.1$, which is activated by the increase in the cytosolic free Ca^{2+} concentration above 200 nM (Panyi et al., 2014). Like in PDAC cancer cells (see *K^+ Channels in Pancreatic Cancer Cells*), these K^+ channels maintain a permissive negative membrane potential for efficient Ca^{2+} signaling, and their inhibition interferes with T-cell activation (Panyi et al., 2006). This general mechanism is tailored to the T-cell subtypes either by changing the relative expression level of $K_V1.3$ vs. $K_{Ca}3.1$, or by changing the subunit composition of the CRAC channel.

T-cell subsets in PDAC and their corresponding ion channel repertoire. The major antitumor *effector* cells in PDAC belong to $CD4^+$ helper and $CD8^+$ cytotoxic cells, usually present in the tumor mass in similar proportions (Carstens et al., 2017; Stromnes et al., 2017). $CD4^+$ helper T-cells are actually a broad composition of several subtypes (mainly T_h1, T_h2, and T_h17). The 20-year-old theory of the so-called "T_h1/T_h2 balance" stated that the cancerous environment causes a decrease in the T_h1/T_h2 ratio, toward a T_h2-dominated and protumoral condition (Shurin et al., 1999). Also in PDAC, the helper T-cell ratio is pivotal (Wörmann et al., 2014). Thus, T_h2-related cytokines like IL-4 (Piro et al., 2017) or IL-6 (Mroczko et al., 2010) have been deemed as "prognostic" by many researchers (De Monte et al., 2011; Yako et al., 2016). T_h17 cells have an uncertain position in the tumor milieu, although the

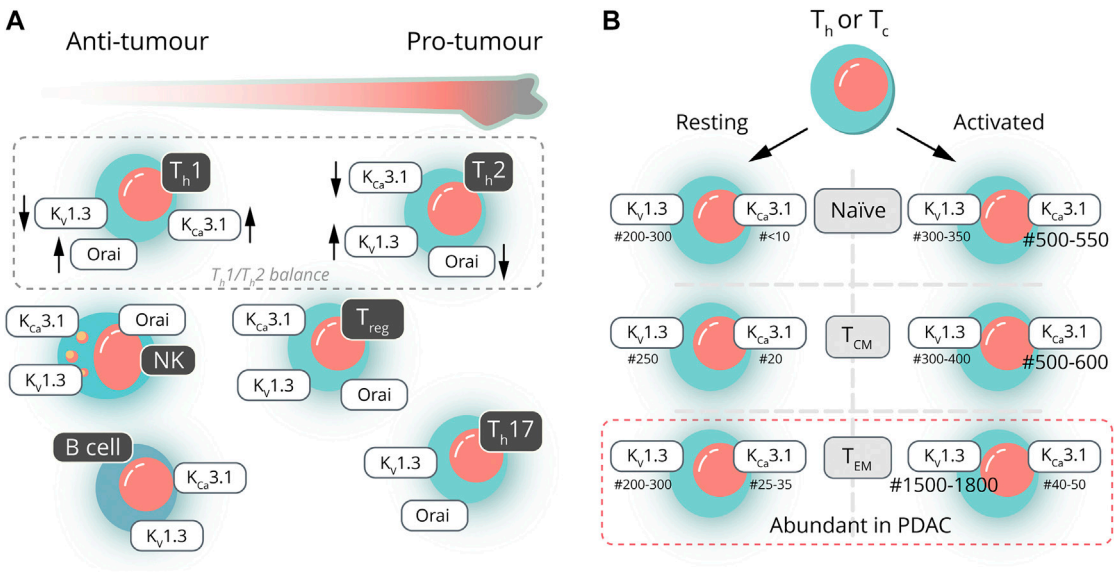


FIGURE 2 | Ion channels in pancreatic ductal adenocarcinoma—infiltrating lymphocytes. **(A)** Lymphocytes found in the PDAC microenvironment can be either pro- or antitumorigenic. The ratio of T-helper lymphocytes (T_h1/T_h2; dashed line) has a prognostic value in assessing therapy outcome. The presence of other lymphocytes can be either beneficial (NK and B cells) or detrimental (T_{reg} and T_h17) (yet, not univocally). These PDAC-infiltrating lymphocytes often show distinct channel activities, which could be considered in targeted PDAC therapies. **(B)** Both helper (T_h) and cytotoxic (T_c) T lymphocytes can be further subdivided into naive, central memory ($T_{C,M}$) or effector memory (T_{EM}) T lymphocytes, the latter being the most abundant subtype in PDAC (indicated by the dashed line). Activation of T lymphocytes leads to characteristic changes in the numbers (#) of $K_{Ca}3.1$ and $K_V1.3$ channels. Activation of naive and T_{CM} is associated with an increase in $K_{Ca}3.1$ expression, whereas activation of T_{EM} causes a distinct increase in the number of $K_V1.3$ channels.

majority of cases tends toward a protumoral effect (Murugaiyan and Saha, 2009; Ivanova and Orekhov, 2015). IL-17 and IL-22, produced by T_h17 cells, are correlated with a bad prognosis in PDAC (McAllister et al., 2014; Wen et al., 2014). Contrariwise, using the murine cancer cell line PANC-02 and inducing T_h17 function produced an antitumor effect (Gnerlich et al., 2010).

When naive T-cells encounter their specific antigens, they differentiate into central memory T-cells (T_{CM}) and effector memory T-cells (T_{EM}) of either $CD4^+$ or $CD8^+$ phenotype. The majority of T-cells in an orthotopic mouse PANC02 PDAC model are effector/effector memory T-cells (Shevchenko et al., 2013; Bazhin et al., 2016). In a similar fashion, in human PDAC, most $CD8^+$ tumor-infiltrating lymphocytes are effector memory cells (Poschke et al., 2016; Stromnes et al., 2017).

As noted above, relatively little is known about the ion channel expression pattern in various T-cell subsets infiltrating the PDAC tissue. Compared to naive T-cells, rich in both Orai1 and Orai2, effector $CD4^+$ and $CD8^+$ T-cells down-regulate only Orai2, generating more Orai1 homohexamers, which are characterized by a superior Ca^{2+} conductance. In this manner, effector T-cells allow larger Ca^{2+} influxes and activate quicker after the antigen recognition than naive cells (Vaeth et al., 2017). Naive human $CD4^+$ and $CD8^+$ T lymphocytes are characterized by 200–300 $K_V1.3$ and less than 10 functional $K_{Ca}3.1$ channels, whereas their activated counterparts mildly up-regulate $K_V1.3$ (300–350 channels/cell) and severely increase $K_{Ca}3.1$ expression (500–550 channels/cell) (Ghanshani et al., 2000; Wulff et al., 2003; Cahalan and Chandy, 2009).

Murine $CD4^+$ T_h1 and T_h2 have similar numbers of $K_V1.3$ channels in their membranes, while murine T_h2 cells have substantially less functional $K_{Ca}3.1$ channels than T_h1 lymphocytes. This difference in the $K_{Ca}3.1$ expression/function may explain the larger amplitudes of the Ca^{2+} signals in T_h1 cells (Fanger et al., 2000; Di et al., 2010). A flow cytometric analysis of $K_V1.3$ expression in human peripheral blood lymphocytes showed that the T_h1 subset has less $K_V1.3$ channels than T_h2 (Toldi et al., 2011; Orbán et al., 2014). Consistent with this, they also reported that $K_V1.3$ inhibitors have a smaller impact on the Ca^{2+} transients in T_h1 lymphocytes than $K_{Ca}3.1$ inhibitors (Toldi et al., 2011). Moreover, Ca^{2+} influx through CRAC is more prevalent in T_h1 than T_h2 cells (Toldi et al., 2012). Both mouse and human T_h17 lymphocytes have the highest $K_V1.3$ expression, but no or little $K_{Ca}3.1$ expression (Di et al., 2010; Orbán et al., 2014). It should be pointed out that the role of $K_V1.3$ and $K_{Ca}3.1$ in regulating Ca^{2+} signaling in human T-cell subsets, addressed in the articles above, has not yet been confirmed using electrophysiology.

T_{CM} and T_{EM} memory cells not only differ in characteristic membrane markers and in their homing and trafficking ability but also in their ion channel repertoire. Human resting T_{CM} and T_{EM} cells, whether they are $CD4^+$ or $CD8^+$, are similar to naive cells regarding their $K_V1.3$ and $K_{Ca}3.1$ expression. When activated, they dichotomically diverge: T_{CM} cells show the usual $K_{Ca}3.1^{high}$ phenotype, whereas T_{EM} cells exhibit a dramatically increased $K_V1.3$ expression in the plasma membrane (Ghanshani et al., 2000; Wulff et al., 2003; Cahalan and Chandy, 2009). (For more details with respect to the channel expression of the various subtypes, see **Figure 2B**.)

B lymphocytes in PDAC and their corresponding ion channel repertoire. The role of B cells in the pathology of PDAC is not well defined. In human PDAC, they are associated with a good prognosis (Castino et al., 2016; Brunner et al., 2020). However, this is not adequately mirrored by the existing mouse models (Spear et al., 2019). In 2016, three different research groups showed that B cells have a protumorigenic role in genetically modified mice (KC mice expressing the oncogenic Kras in the pancreas only) and in healthy mice orthotopically injected with KC cells (reviewed in Roghanian et al. (2016)). KMC, a mouse model characterized by the knock-in of one or two copies of *Myc* (Farrell et al., 2017), develops one of the most aggressive and histologically human-like PDAC. It is not as strongly infiltrated by NK and B cells as the slowly developing KC model. The removal of the *Myc* gene promotes NK and B cells to enter the tumor mass, lengthening the life span of mice (Muthalagu et al., 2020). Some studies merely report B-cell infiltration in PDAC based on their CD20 expression (Brunner et al., 2020), and further classification of the cells based on their activation status (i.e., CD27 expression) is lacking. Other studies, for example, Castino et al., found that interspersed B cells from PDAC show little or no CD27 and other fundamental markers. However, when they organize themselves in tertiary lymphoid tissue structures within the tumor stroma, CD27 expression is up-regulated (Castino et al., 2016).

There is a strong relationship between the ion channel expression and the activation status of the B cells (Wulff et al., 2004). Human naive (IgD^+ $CD27^-$) and early memory (IgD^+ $CD27^+$) B cells, just like naive and central memory T-cells, are abundant in $K_V1.3$ and virtually lack $K_{Ca}3.1$ (naive: $K_V1.3$: 90–100, $K_{Ca}3.1$: 5 channels/cell; early memory: $K_V1.3$: 250, $K_{Ca}3.1$: 5–10 channels/cell). These cells, when activated, overexpress only $K_{Ca}3.1$ channels (naive: $K_V1.3$: 80–100, $K_{Ca}3.1$: 550–650 channels/cell; early memory: $K_V1.3$: 150–200; $K_{Ca}3.1$: 650–750 channels/cell) (Wulff et al., 2004; Cahalan and Chandy, 2009). Late memory class-switched B cells (IgD^-CD27^+) have plenty of $K_V1.3$ and few $K_{Ca}3.1$ ($K_V1.3$: 2,200–2,600; $K_{Ca}3.1$: 50–70 channels/cell). They tend to further enhance their $K_V1.3$ expression when activated ($K_V1.3$: 2,900–3,300; $K_{Ca}3.1$: 60–80 channels/cell).

Adaptive Immunity: Protumor Cells

Regulatory T-cells in PDAC and their corresponding ion channel repertoire. Human T_{reg} cells are important immunosuppressive $CD4^+$ lymphocytes heavily implicated in autoimmunity. They are usually identified by the signature $CD4^+CD25^+Foxp3^+$ (Whiteside, 2015). In contrast to the healthy pancreas (Weisberg et al., 2019), PDAC is heavily infiltrated by T_{reg}s. They can reach 20–40% of the whole $CD4^+$ pool (Shevchenko et al., 2013; Tang et al., 2014; Bazhin et al., 2016). Recently, it has been discovered that PDAC tissues overexpress a cancer-related Foxp3 protein (c-Foxp3) which, through the secretion of CCL5, would recruit a high number of T_{reg} cells in the tumor environment (Wang et al., 2017).

Similarly to their helper $CD4^+$ counterpart, human T_{reg}s also have a high number of voltage-gated $K_V1.3$ channels (Estes et al., 2008; Shao et al., 2018) and a low number of $K_{Ca}3.1$ channels in their membrane (Varga et al., 2009). Intratumoral T_{reg}s have a peculiar T-cell receptor repertoire whose stimulation may have an important role in their immunosuppressive function (Ahmadzadeh et al., 2019). When human T_{reg}s get activated by T-cell receptor stimulation, they do not up-regulate $K_V1.3$ as normal effector T-cells do (Reneer et al., 2011) and activated human T_{reg}s incubated with $K_V1.3$ and $K_{Ca}3.1$ blockers do not show any difference in the Ca^{2+} influx, suggesting that the contribution of these channels to the activation could be minimal (Orbán et al., 2014). Moreover, T_{reg}s from $K_{Ca}3.1$ knockout mice are able to suppress T-cell proliferation in a comparable manner as wild-type T_{reg}s, pointing to a minor role of these channels (Di et al., 2010). Similarly, knocking out $K_V1.3$ in mice does not hinder the physiological generation of T_{reg}s (Gocke et al., 2012). Hence, the role of these K^+ channels in T_{reg}s is currently still unclear. CRAC channels are involved in T_{reg} development and contribute to their suppressive function (Vaeth et al., 2019).

THERAPEUTICAL APPROACHES AND ION CHANNELS IN PDAC

Unfortunately, PDAC therapy has remained largely ineffective. Radical surgical resection of the tumor as well as chemotherapeutic agents like gemcitabine combined with nab-paclitaxel and FOLFIRINOX constitutes the standard therapy for PDAC patients (Hessmann et al., 2020). However, 80–90% of the patients present at an advanced unresectable stage at the time of diagnosis. Even if surgical intervention is possible, recurrence of the cancer lesions will be common (Vincent et al., 2011; Peixoto et al., 2015; Rawla et al., 2019). Therapy resistance is in part due to the fibrotic microenvironment in PDAC which hinders drugs from reaching their target and due to the immunosuppressive properties of the PDAC tumor microenvironment. Nonetheless, so far, therapeutic targeting of the PDAC tumor microenvironment has not been successful (reviewed in Hessmann et al. (2020)).

Electrolytes and Organic Metabolites in PDAC

The great majority of the studies using ion channel blockers or activators are aimed to target cancer cells, rather than immune or stroma cells. The therapeutic potential of ion channels of cancer-associated immune and stroma cells has not been analyzed in great detail so far. In a groundbreaking study by Eil et al., it was shown that B16 (mouse) or Mel624 (human) melanoma cells subcutaneously injected into mice create a TME much richer in K^+ than serum (40 mM vs. 5 mM), probably due to marked necrosis within the tumor (Eil et al., 2016). Since more than 60% of PDAC cases host micro- and/or macronecrotic spots (Hiraoka et al., 2010), it is plausible to hypothesize that PDAC is a tumor rich in extracellular K^+ as well. The T-cell $[K^+]_i$ is around 130 mM, but when cultured in a medium with high $[K^+]_o$—specular of what happens *in vivo*- the $[K^+]_i$ in these cells can rise above

150 mM (Eil et al., 2016; Ong et al., 2019). Although the $[K^+]$ changes would result in the depolarization of the cell membrane, the authors did not find a diminished Ca^{2+} influx into the T-cells during their activation in high $[K^+]_o$ (Eil et al., 2016). This is opposite to the generally accepted role of the membrane potential in controlling Ca^{2+} signaling.

Membrane depolarization could also lead to enhanced IL-2 signaling in T_{reg}s and, consequently, to suppressed antitumor immune surveillance by such an unbalanced ionic environment (Nagy et al., 2018). The high $[K^+]$-adapted tumor-infiltrating lymphocytes are less functional than the normal ones. Conversely, decreasing the $[K^+]_i$ in a forced manner using the Na^+/K^+ ATPase blocker ouabain renders the $CD8^+$ cells more functional again. Moreover, substituting normal (poorly functioning) immune cells with $CD8^+$ cells overexpressing the K^+ efflux channels $K_V1.3$ or $K_{Ca}3.1$ boosts their antitumor activity. Tumor growth is slowed down, and this improves the survival (Eil et al., 2016). These findings nicely illustrate how ion channel function and thereby cell function depend on the "correct" ionic composition of the pericellular environment.

The disrupted ion balance in the tumor microenvironment also affects the operation of protumor immune cells. Increasing $[Na^+]_o$ and $[Cl^-]_o$ in melanoma as well as lung and breast cancer by the administration of a high salt diet (HSD) inhibits the capacity of MDSCs to suppress antitumor cytotoxic cells. Accordingly, this treatment has made the tumors to shrink in size (Willebrand et al., 2019; He et al., 2020). High $[Na^+]_o$ and $[Cl^-]_o$ partially inhibit the function of thymus-derived mouse T_{reg}s as well (Luo et al., 2019), which could also contribute to the less immunosuppressive environment. Whether these electrolytes influence antitumor $CD4^+$ and $CD8^+$ T-cells is unclear. Melanoma and breast cancer growth in BABL/C-nu/nu mice, lacking sufficient T-cell–mediated immune reactions, are insensitive to a high salt diet, indicating that T-cell–mediated antitumor response is key to the high salt diet–induced antitumor activity (He et al., 2020). On the other hand, the high salt diet prevents tumors from growing also in $RAG2^{-/-}$ mice, despite the lack of T and B cells in these animals. This suggests that the high salt diet may act by yet another mechanism independent of immune cell modulation (Willebrand et al., 2019). All these studies clearly show that the electrolyte imbalance in the tumor microenvironment significantly contributes to tumor progression and may shape the immune response.

High salt diet increases the osmolarity in the cancer tissue (He et al., 2020), which may influence the volume regulation of immune and cancer cells and link the high–salt diet effects described above to ion channels. Cell volume changes are known to regulate cancer cell migration, invasion, and apoptosis in an ion channel (and transporter)–dependent manner (see for reviews (Bortner and Cidlowski, 2014; Schwab and Stock, 2014)). In addition, cell volume changes in immune cells regulate apoptosis (Bortner and Cidlowski, 2011) and B-cell activation (Cvetkovic et al., 2019). Although the pivotal role of ion channels in volume regulation in immune (Feske et al., 2015) and cancer cells (Morishita et al., 2019) is well established, the relevant ion channels that sense the altered ion concentrations and osmolarity in the PDAC microenvironment are yet to be identified.

In addition to $[K^+]$, the pH in the tumor microenvironment also changes characteristically during the progression of the disease. The nondiseased pancreas stroma is deemed to endure postprandial tides of acidic pH in order to counterbalance the apical excretion of HCO_3^- ions. During the development of PDAC, the tumor microenvironment becomes very acidic (Pedersen et al., 2017). The extra- and intracellular pH regulate voltage-gated K^+ channels of the K_V1 Shaker family (Starkus et al., 2003). One of the major K^+ channels in lymphocytes, $K_V1.3$, in particular, is uniquely modulated by acidification. A decrease in both pH_i and pH_o reduces the peak $K_V1.3$ current, and acidification of the extracellular medium slows inactivation kinetics of the current (Deutsch and Lee, 1989). This dual regulation of $K_V1.3$ by pH_o allows very sensitive modulation of the K^+ conductance of the membrane. Acidification-induced K^+ current inhibition is counterbalanced, depending on pH_o, by the concomitant slowing of the inactivation kinetics. Interestingly, the slowing of the inactivation kinetics at low pH_o is reverted to acceleration of the kinetics when acidic extracellular pH is combined with elevated $[K^+]_e$ (Somodi et al., 2008). The unique response of $K_V1.3$ to extracellular acidification is mediated by reversible protonation of H399 in the external vestibule of the channel guarding the selectivity filter (Somodi et al., 2004). The protonation of H399 also bears significance on the targeting of the $K_V1.3$ channel by peptide and nonpeptide inhibitors as well. For example, tetraethylammonium (Somodi et al., 2004) and peptide blockers (Rodrigues et al., 2003) lose their affinity for $K_V1.3$, when H399 is protonated. This molecular information about $K_V1.3$ gating at various pH and K^+ concentrations may contribute to the understanding of how the tumor microenvironment interacts with immune cells at the level of ion channels and may allow proper therapeutic ion channel targeting. We refer to our recent review for a more detailed discussion on how cancer progression may be affected by modulation of cation channels in tumor and tumor stroma cells (Pethő et al., 2020).

The disbalance of the tumor microenvironment is not restricted to inorganic electrolytes. It is well known that tumors are rich in ATP and adenosine, which influence cells and their function through purinergic receptors (Feng et al., 2020). The ATP concentration in the interstitial tissue of PDAC is almost 100 times higher than normal pancreatic stroma (~10 μM vs. 100 nM (Hu et al., 2019a)). PDAC cells usually overexpress both CD39 (Künzli et al., 2007) and CD73 (Harvey et al., 2020), which transform ATP to AMP and AMP to adenosine. Thus, it is plausible that PDAC is rich in adenosine as well, which would activate the receptor $A_{2A}R$ and inhibit $CD8^+$ cells via PKA (Chimote et al., 2013). As more thoroughly described later, adenosine also decreases the $K_{Ca}3.1$ conductance of T-cells without affecting the number of channels in the membrane (Chimote et al., 2018). Adenosine exerts *in vivo* and *in vitro* an anti-PDAC effect in immunodeficient nu/nu mice (Yang et al., 2019). This means that the outcome of the adenosine action will be the combination

of inhibiting antitumor immunity and the growth of PDAC. Three clinical trials are currently assessing whether antiadenosine therapy coupled with immuno- and chemotherapies is able to halt PDAC progression (Singh and O'Reilly, 2020).

Pharmacological Targeting of Ion Channels in PDAC

Ion channel targeting is a well-established therapeutic concept in other medical disciplines that has been successfully applied in the clinical routine for decades. Because of their expression in the plasma membrane, ion channels are easily druggable. Intriguingly, many of the ion channels that are known to drive tumor progression are targeted by drugs currently used for noncancer indications, either as the primary effect or as a side effect. These drugs could in principle be repurposed for cancer treatment as suggested for nontorsadogenic $K_V11.1$ blockers (Kale et al., 2015; Pointer et al., 2017). The fact that cancer, stroma, and immune cells rely nearly on the same set of ion channels makes the selection of the right drug a complex quest. Ideally, channel targeting should elicit synergistic effects such as inhibition of tumor and stoma cell proliferation or migration as well as activation of antitumor immune cells or inhibition of tumor-promoting immune cells. This is further complicated by the dynamics/variability of channel expression: in lymphocytes, for example, such expression strongly relies on the activation state of the respective subtype. Moreover, specificity of the channel modulators constitutes another challenge which has not been solved satisfactorily for many channels. However, an increasing number of ion channel protein structures is becoming amenable for *in silico* drug design (e.g., Brömmel et al. (2020b)). Alternatively, peptide-based blockers or antibody targeting could be more specific alternatives (Duranti and Arcangeli, 2019; Hartung et al., 2020; Tajti et al., 2020). Here, we list potential target ion channels and the existing knowledge about the consequence of their activation/inhibition in immune, stroma, and tumor cells as well.

$K_{Ca}3.1$ is a well-studied channel in this context. The specific modulators of $K_{Ca}3.1$ are inhibitors, such as TRAM-34 (Wulff et al., 2000), senicapoc, NS6180, and verapamil (Wulff et al., 2007), and activators such as riluzole (Liu et al., 2013) and 1-EBIO (Devor et al., 1996). Molecular modeling suggests that senicapoc binds to $K_{Ca}3.1$ channels in the open conformation (Brömmel et al., 2020a). The inhibition or activation of an ion channel may result in diminished or augmented cellular functions. For example, two specific inhibitors of $K_{Ca}3.1$ (TRAM-34 and NS6180) boosted *in vivo* adherent human NK-cell proliferation, degranulation, and capacity of killing erythroleukemic cells in mice (Koshy et al., 2013). Conversely, activation of $K_{Ca}3.1$ channels with 1-EBIO inhibits migration of transformed renal epithelial cells almost as effectively as channel inhibition (Schwab et al., 2006). As discussed above, T lymphocytes in PDAC are supposedly fairly devoid of $K_{Ca}3.1$; hence, the use of such a blocker should not hinder physiological function of these cells, regardless of $[K^+]$ in the tumor microenvironment. On the other hand, in mice, the adoptive cell transfer of T_{CM} cells, a minority in PDAC but rich in $K_{Ca}3.1$, more efficiently combats melanoma than the transfer of the T_{EM} subset that poorly expresses $K_{Ca}3.1$ (Klebanoff et al., 2005, 2016). Unfortunately, the consequence of the modulation of $K_{Ca}3.1$ activity of T_{EM} and T_{CM} cells has not been elaborated yet in tumor models.

$K_{Ca}3.1$ is unique among the K^+ channels of the immune system since the consequences of both channel inhibition and activation can be studied. Activation of $K_{Ca}3.1$ by 1-EBIO is able to induce movement in akinetic $CD8^+$ cells isolated from the peripheral blood of head and neck squamous cell carcinoma patients and ameliorate their IFN-γ production (Chimote et al., 2018). Thus, pharmacological activation of $K_{Ca}3.1$ may enhance antitumor activity of $CD8^+$ T-cells by overcoming the inhibition of the $K_{Ca}3.1$ activity caused by large amounts of adenosine in cancer stroma and/or localized down-regulation of membrane-proximal calmodulin, the Ca^{2+} sensor of $K_{Ca}3.1$. The diminished association of calmodulin with the channel suppresses the $K_{Ca}3.1$ activity in circulating T-cells and limits their ability to infiltrate adenosine-rich tumor-like microenvironments (Chimote et al., 2020). How NK cells would behave with a $K_{Ca}3.1$ activator has not been studied. It is known however that in patients suffering from amyotrophic lateral sclerosis, riluzole, which is the only disease-modifying therapy for ALS, does not compromise NK cells from entering the spinal cord and the motor cortex (Garofalo et al., 2020). Based on this, we can hypothesize that $K_{Ca}3.1$ activation would not interfere with the antitumor action of NK cells.

In vitro studies also demonstrated that the $K_{Ca}3.1$ activator SKA-346 increases the IFN-γ secretion of high $[K^+]$-inhibited human T-cells by 50% (Ong et al., 2019). Neither this nor new $K_{Ca}3.1$ activators, like SKA-31 and SKA-121 (Ohya and Kito, 2018), have been tested in PDAC; hence, their usefulness is, in light of our current knowledge, unknown.

It is important to point out that $K_{Ca}3.1$ activators are also targeting cancer cells and stromal cells. This has been shown for multiple cancer entities (see Mohr et al., (2019) for review) including PDAC (Jäger et al., 2004; Bonito et al., 2016; Storck et al., 2017). Riluzole and SKA-31 were successfully used to suppress the expansion of colorectal cancer cell lines, in combination with the $K_V11.1$ channel blocker E4031 (Pillozzi et al., 2018) (see below for a detailed discussion of the challenges associated with targeting K_V11 channels in PDAC therapy). Another study by Sun et al. showed that riluzole is capable of inhibiting the proliferation and partly killing several human PDAC cell lines (Sun et al., 2019). These effects, in combination with the aforementioned effect of $K_{Ca}3.1$ activators in rebalancing T-cell $[K^+]_i$, could synergistically act in the treatment of malignancies by targeting the $K_{Ca}3.1$ channel.

Although riluzole has been defined and used as a $K_{Ca}3.1$ activator (Pillozzi et al., 2018), this has to be viewed with caution since its specificity is very limited and it inhibits a series of other ion channels, such as $K_V11.1$ (Pillozzi et al., 2018) and, most notably, several voltage-gated sodium channels (**$Na_V1.x$**) (Song et al., 1997; Djamgoz and Onkal, 2012). Several human carcinomas express those Na_Vs, which promote invasiveness and metastasis under hypoxic conditions (Djamgoz et al., 2019). Inhibition of the hypoxia-sensitive

persistent component of the Na_V current (INa_P) by riluzole suppresses cancer cell invasiveness *in vitro* (Djamgoz and Onkal, 2012). There is evidence that aggressive PDAC cell lines express $Na_V1.5$ and $Na_V1.6$, but these channels do not seem to play a major functional role in PDAC cells. Na_V channel activity becomes measurable only upon EGFR inhibition (Bonito, 2017). Hence, we can suppose that riluzole will not influence PDAC growth by its direct action on Na_V. The aforementioned effects of the "broad-spectrum" ion channel inhibitor riluzole on PDAC cell lines (Sun et al., 2019) are therefore more likely to be attributed to its action on the $K_{Ca}3.1$ or $K_V11.1$ channels. Nonetheless, the use of a broad-spectrum ion channel modulator can be justified when it yields the desired phenotypic effects (e.g., inhibition of migration and proliferation). Phenotypical drug screening is at least as successful as molecular screening which aims at a single molecularly defined target (Zheng et al., 2013).

Riluzole has pleiotropic effects, including dampening of excitotoxicity through interruption of glutamatergic transmission in the central nervous system (Benavides et al., 1985). This effect may be mediated by the inhibition of the activation of NMDA glutamate receptors (Debono et al., 1993). In addition to the inhibition of glutamatergic transmission, the beneficial effects of riluzole in the treatment of ALS may include inhibition of presynaptic voltage-gated Ca^{2+} channels and the inhibition of Na_V channels and block of the persistent Na^+ currents in motoneurons ((Lamanauskas and Nistri, 2008), and see Cheah et al. (2010) for review). Thus, the use of riluzole as a $K_{Ca}3.1$ activator may be limited by its effects on the central nervous system.

The action of Na_V inhibitors is further complicated as human immature dendritic cells express $Na_V1.7$ (Zsiros et al., 2009). Silencing $Na_V1.7$ shifts the membrane potential to more hyperpolarized values in $CD1a^+$ immature dendritic cells. This results in decreased cell migration, which otherwise is a hallmark of a functionally immature dendritic cells (Kis-Toth et al., 2011). How this would impact in PDAC, a tumor known for its paucity in dendritic cell infiltration (Hegde et al., 2020), has not been explored yet.

The ATP-rich PDAC environment could also enhance immunosuppression through **P2X7**, which is present in both T lymphocytes (Feske et al., 2012) as well as in cancer (Giannuzzo et al., 2015) and stellate cells in PDAC (Haanes et al., 2012). This cation-selective ion channel is important for the infiltration of T lymphocytes into the tumor, since $P2X7^{-/-}$ mice have a suboptimal immune response (De Marchi et al., 2019). Interestingly, inhibiting P2X7 with a selective antagonist like A740003 causes an increase in the percentage of infiltrating $CD4^+$ cells and a significant drop in tumor weight and T_{reg} abundance (De Marchi et al., 2019). Whether these results, obtained with a mouse melanoma model, can be translated to immune modulation in a "cold" tumor like PDAC is unclear: the few reports about *in vivo* administration of P2X7 antagonists (AZ10606120 and A438079) did not analyze the involvement of the immune system (Mohammed et al., 2017) or used immunodeficient nude mice (Giannuzzo et al., 2016).

Challenges of Using K_V11 Channels as Therapeutic Target in PDAC

The most critical issue for using $K_V11.1$ channel blockers in PDAC therapy have and still will be their cardiac side effects. Indeed, $K_V11.1$ channels are highly expressed in the heart, representing the molecular correlate of the rapid repolarizing current I_{Kr} (Sanguinetti et al., 1995; Trudeau et al., 1995). Due to their peculiar gating properties, these channels are very effective in sustaining fast repolarizations in cardiac myocytes (Sanguinetti, 2010). For these reasons, pharmacological inhibition or malfunction of the $K_V11.1$ channel can lead to potentially life-threatening arrhythmias, such as the long QT syndrome (LQT) (Sanguinetti, 2010; Mitcheson and Arcangeli, 2014).

Many $K_V11.1$ channel blockers like the methanesulfonanilide E4031, which belong to class III of antiarrhythmic drugs (Ågren et al., 2019), require an open channel to gain access to the channel pore. They block the K^+ flow and hence can delay heart repolarization (Mitcheson and Arcangeli, 2014; Chen et al., 2016), thus lengthening the QT interval. The capacity of different compounds to block $K_V11.1$ channels and induce QT prolongation (the so-called "QT liability") differs among structurally diverse compounds (Sanguinetti, 2010; Authier et al., 2017) so that some drugs effectively block $K_V11.1$ channels but do not cause arrhythmias. These drugs are addressed as "nontorsadogenic $K_V11.1$ blockers." These drugs may represent good candidates for anticancer therapy (Cernuda et al., 2019).

An example is represented by *R*-roscovitine, a cyclin-dependent kinase (CDK) inhibitor that, although blocking $K_V11.1$ currents in an open channel manner, shows no use dependency. This suggests rapid block and unblock kinetics (Cernuda et al., 2019). Thus, R-roscovitine could be used to target $K_V11.1$ for PDAC therapy. Besides this compound, other studies have pointed out a potential for many "nontorsadogenic" $K_V11.1$ blockers (Pointer et al., 2017) and drugs which bind the $K_V11.1$ channel in a specific conformation (Becchetti et al., 2019). For example, the macrolide antibiotic clarithromycin, which is commonly used for bacterial infection, such as *H. pylori*, binds to $K_V11.1$ in the closed conformation and shows a good antitumor activity in colorectal cancer (Petroni et al., 2020). Because it is already in clinical use for bacterial infections, it could also potentially serve as an antitumor drug in epithelial cancers like colorectal cancer and PDAC, as already shown in lymphomas (Ferreri et al., 2018).

Another approach is to target the $K_V11.1$ subunit trafficking to the membrane. Up to now, several molecules have been shown to interfere with trafficking without having any effect on the channel itself. These include arsenic trioxide, geldanamycin, pentamidine, and probucol (Chen et al., 2016). Notably, arsenic trioxide has been and is still used as an anticancer drug, especially in leukemias (Hoonjan et al., 2018).

Yet another approach is to target $K_V11.1$ channels with antibodies. The clinical potential of monoclonal antibodies has emerged in the last decade. However, due to the cardiovascular side effects seen for many drugs, targeting the $K_V11.1$ channel with single-chain function blocking antibodies is also challenging and not preferred. $K_V11.1$ channel–specific antibodies, such as the single-chain variable fragment (scFv), anti-Kv11.1 ScFv, are more relevant

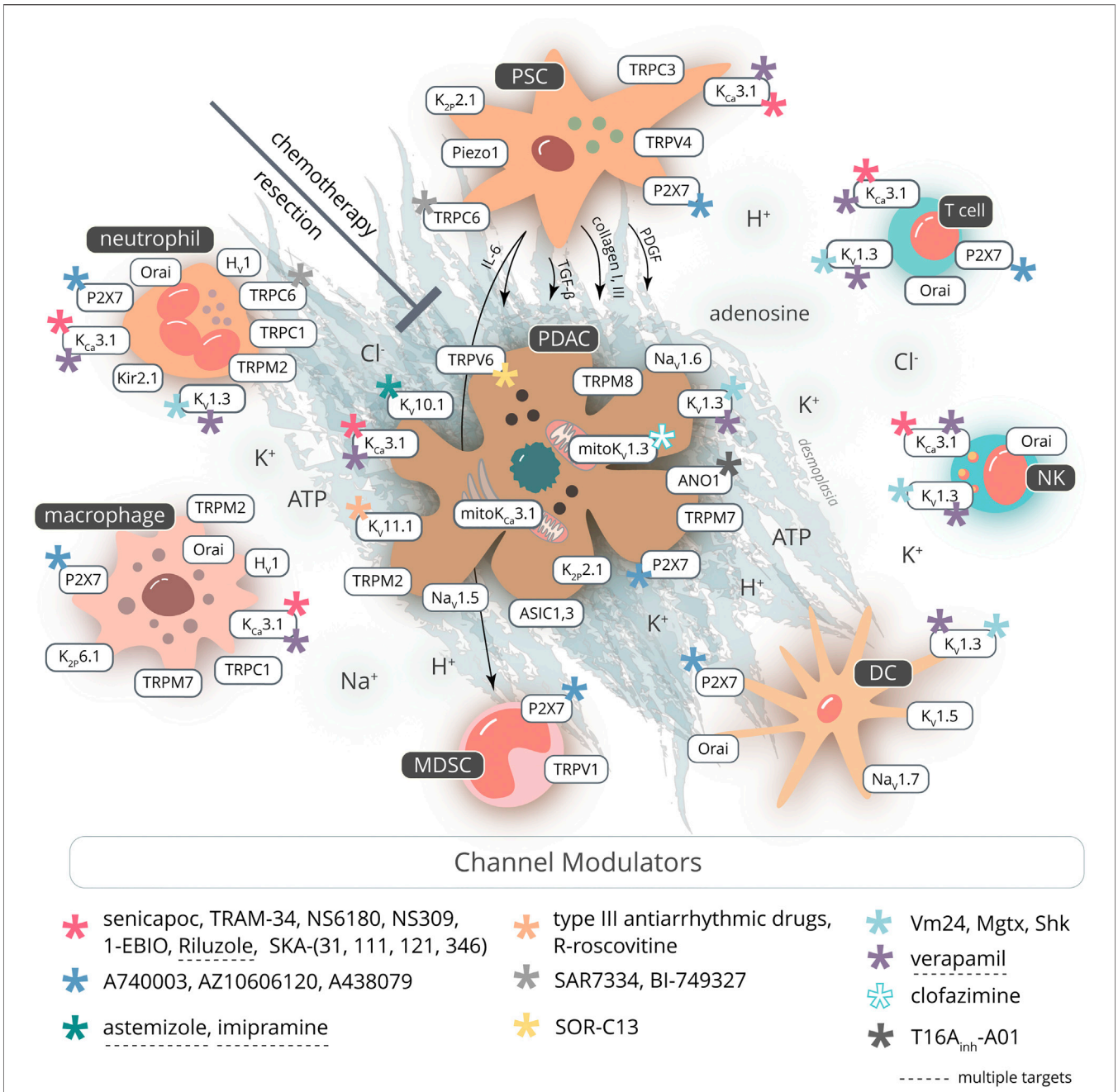


FIGURE 3 | Ion channels in pancreatic ductal adenocarcinoma microenvironment and examples of channel modulators. Up to date, resection and chemotherapy are the only therapeutic approaches against PDAC. However, the fibrotic and acidic immunosuppressive tumor milieu hinders drug delivery and disturbs immune response. Ion channels expressed in tumor, stromal, and immune cells control cellular responses and therefore stand for putative drug targets. Here, we display those channels whose function has been investigated in cells of the PDAC tumor tissue. The respective modulators are indicated by asterisks.

for cancer diagnosis, rather than for cancer treatment (Duranti et al., 2018). The risk of cardiac side effects can be reduced by employing bispecific antibodies. $K_V11.1$ interacts with β1 integrins in PDAC, which is a tumor-specific feature of $K_V11.1$ channels (Arcangeli and Becchetti, 2017). A convenient approach would therefore be to target this complex with a bifunctional single-chain diabody (scDb). This would increase the antibody–cancer cell specificity and reduce cardiovascular side effects. It may be taken as a proof of principle for therapeutic ion channel targeting that a monoclonal antibody against $K_V10.1$ reduces tumor volume in a human pancreatic cancer xenograft mice model (Gómez-Varela et al., 2007).

Finally, antibody–drug conjugates (ADC) are biopharmaceutical drugs that are used for targeted therapy (e.g., Pahl et al. (2018)). The combination of cancer specificity

and cytotoxicity makes them good candidates for next-generation cancer therapy (Parslow et al., 2016). An anti-K_V11.1/β1 scDb could potentially be linked to cytotoxic drugs to create highly specific ADCs for pancreatic cancer treatment.

OUTLOOK

It remains an unsolved challenge to provide PDAC patients with an efficient therapy. New therapeutic strategies are urgently needed since none of the current treatment options yields satisfactory results. Presently, it is controversially debated whether the desmoplastic PDAC stroma and microenvironment can be targeted therapeutically. The conflicting results of these studies indicate that the properties and function of stromal cells such as pancreatic stellate cells and PDAC-infiltrating immune cells are far from being fully understood. In our view, an important class of membrane proteins has been neglected in this context: ion channels. They have been shown to be important drivers of aggressive cancer cell properties and related to all hallmarks of cancer. Being membrane proteins, ion channels are in a position to modulate, sense, and transduce properties of the tumor microenvironment. However, their roles in pancreatic cancer cells and in cells of the PDAC microenvironment are largely unexplored.

We collected the current knowledge of ion channels in PDAC with a particular focus on immune and stromal cells and discussed the feasibility of therapeutic ion channel targeting. **Figure 3** gives a graphical overview. Many tumors (mis-)use ion channels as highlighted for PDAC in this review. Based on the rapidly growing mechanistic knowledge about ion channels in PDAC, we are convinced that ion channel targeting offers a chance for therapeutic intervention.

We conclude that the choice of the right blocker or activator must be accurately rationalized, at least until we obtain enough data from electrophysiological measurements. Such data are still lacking for PDAC-infiltrating immune cells (Panyi et al., 2014). Similarly, there is hardly any electrophysiological data on stromal cells like PSCs. Studying ion channel activity directly in tumor-derived immune or stromal cells is an important issue. Their channel expression could show distinct differences from that of peripheral blood mononuclear cells or stromal cells isolated from a healthy pancreas. Moreover, it is imperative to consider the microenvironmental conditions under these circumstances. Up-regulation of channel expression may well be counterbalanced by characteristic properties of the tumor microenvironment such as the tumor acidity (Pethő et al., 2020). Since no K_V1.3 activators are available at the moment (Chandy and Norton, 2016), K_{Ca}3.1 activators appear promising in boosting tumor-infiltrating lymphocytes. Repurposing of riluzole is a good candidate for therapeutic K_{Ca}3.1 activation; however, the lack of selectivity for K_{Ca}3.1 might limit its application. At the same time, K_{Ca}3.1 inhibitors could activate NK cells and stop cancer cells or stromal cells from proliferating and/or migrating. Only *in vivo* studies will reveal in the future which arm of the balance we should put our weights on when developing channel-targeting PDAC therapies. Thus, crossing $K_{Ca}3.1^{-/-}$ mice with transgenic breast cancer mouse models provided evidence that the impact K_{Ca}3.1 targeting in cancer cells may be further modulated by K_{Ca}3.1 targeting in noncancerous cells (Steudel et al., 2017). In this context, the right choice of the animal model is crucial, since mouse lymphocytes possess a different set of ion channels than rat and human ones (Beeton and Chandy, 2005). Such studies also need to evaluate repurposed drugs that are already in clinical use or have been tested in phase III clinical trials for other indications such as the K_{Ca}3.1 blocker senicapoc (Ataga et al., 2011).

Another channel modulator that might serve as a target for drug repurposing is clofazimine. It inhibits K_V1.3, thereby inducing apoptosis in PDAC cells and reducing primary tumor weight *in vivo* (Zaccagnino et al., 2017). It is already used for treatment of autoimmune disease and leprosy (Garrelts, 1991). However, K_V1.3 block, inhibition of Na^+/K^+-ATPase of activated T-cells (Anderson et al., 1986), and the release of ROS and PGE_2 from bystander neutrophils (Anderson et al., 1986) inhibit T-cell proliferation and thus may compromise antitumor immunity as well. Follow-up studies are needed to tackle already existing ion channel modulators (inhibitors or activators) for a fast translation into the clinic for PDAC patients. We anticipate that combining innovative animal models with repurposed ion channel targeting drugs—potentially in combination with existing chemotherapeutic therapies—will open up exciting new options. Clearly, such studies would enormously profit from building larger (inter-)national research consortia that can address the diverse channel-related aspects of PDAC pathophysiology in a concerted manner.

AUTHOR CONTRIBUTIONS

All authors of this review contributed to designing and writing this manuscript.

FUNDING

This work was funded by the Marie Skłodowska-Curie Innovative Training Network (ITN) (grant Agreement number: 813834 - *pHioniC* - H2020-MSCA-ITN-2018). AS thanks the support from the Deutsche Forschungsgemeinschaft (DFG; SCHW 407/17-1 & SCHW 407/22-1; GRK 2515/1, Chembion) and IZKF Münster (Schw2/020/18). The National Research, Development, and Innovation Office, Hungary, grant OTKA K119417 (GP); the Ministry of Human Capacities, Hungary, grant EFOP-3.6.2-16-2017-00006 (GP); and the Ministry of Finance, Hungary grant GINOP-2.3.2-15-2016-00015 (GP) are highly appreciated. The project is cofinanced by the European Union and the European Regional Development Fund. This project was supported by AIRC, Grant N° IG 15627 and IG 21510 to AA, PRIN Italian Ministry of University and Research (MIUR) "Leveraging basic knowledge of ion channel network in cancer for innovative therapeutic strategies (LIONESS)" 20174TB8KW to AA.

ACKNOWLEDGMENTS

The authors wish to thank past and present members of our laboratories whose enthusiastic work made it possible to develop

the concepts described in this review. All the images in **Figure 1** are kind gifts from E. Lastraioli, University of Florence, and Prof. G. Perrone Campus Bio-medico, University of Rome. Finally, we would like to mention that this review helped us keep the spirits up during the COVID-19 pandemic in 2020 when work in our laboratories was severely restrained or impossible.

REFERENCES

Abraham, D. M., Lee, T. E., Watson, L. J., Mao, L., Chandok, G., Wang, H. G., et al. (2018). The two-pore domain potassium channel TREK-1 mediates cardiac fibrosis and diastolic dysfunction. *J. Clin. Invest*. 128, 4843–4855. doi:10.1172/JCI95945

Adapala, R. K., Thoppil, R., Luther, D. J., Paruchuri, S., Gary, J., Chilian, W. M., et al. (2013). Integrating mechanical and soluble signals. *J. Mol. Cell. Cardiol*. 54, 45–52. doi:10.1016/j.yjmcc.2012.10.016.TRPV4

Ågren, R., Nilsson, J., and Århem, P. (2019). Closed and open state dependent block of potassium channels cause opposing effects on excitability – a computational approach. *Sci. Rep*. 9, 1–10. doi:10.1038/s41598-019-44564-x

Ahmadzadeh, M., Pasetto, A., Jia, L., Deniger, D. C., Stevanović, S., Robbins, P. F., et al. (2019). Tumor-infiltrating human $CD4^+$ regulatory T-cells display a distinct TCR repertoire and exhibit tumor and neoantigen reactivity. *Sci. Immunol*. 4, eaao4310. doi:10.1126/sciimmunol.aao4310

Anderson, R., Lukey, P., Van Rensburg, C., and Dippenaar, U. (1986). Clofazimine-mediated regulation of human polymorphonuclear leukocyte migration by pro-oxidative inactivation of both leukoattractants and cellular migratory responsiveness. *Int. J. Immunopharmacol*. 8, 605–620. doi:10.1016/0192-0561(86)90033-0

Arcangeli, A., and Becchetti, A. (2006). Complex functional interaction between integrin receptors and ion channels. *Trends Cell Biol*. 16, 631–639. doi:10.1016/j.tcb.2006.10.003

Arcangeli, A., and Becchetti, A. (2017). hERG channels: from antitargets to novel targets for cancer therapy. *Clin. Cancer Res*. 23, 3–5. doi:10.1158/1078-0432.CCR-16-2322

Ataga, K. I., Reid, M., Ballas, S. K., Yasin, Z., Bigelow, C., James, L. S., et al. (2011). Improvements in haemolysis and indicators of erythrocyte survival do not correlate with acute vaso-occlusive crises in patients with sickle cell disease: a phase III randomized, placebo-controlled, double-blind study of the Gardos channel blocker senicap. *Br. J. Haematol*. 153, 92–104. doi:10.1111/j.1365-2141.2010.08520.x

Authier, S., Pugsley, M. K., Koerner, J. E., Fermini, B., Redfern, W. S., Valentin, J.-P., et al. (2017). Proarrhythmia liability assessment and the comprehensive *in vitro* Proarrhythmia Assay (CiPA): an industry survey on current practice. *J. Pharmacol. Toxicol. Methods*. 86, 34–43. doi:10.1016/j.vascn.2017.02.021

Barilla, R. M., Diskin, B., Caso, R. C., Lee, K. B., Mohan, N., Buttar, C., et al. (2019). Specialized dendritic cells induce tumor-promoting IL-10^+ IL-17^+ FoxP3^{neg} regulatory $CD4^+$ T-cells in pancreatic carcinoma. *Nat. Commun*. 10, 1424. doi:10.1038/s41467-019-09416-2

Bauer, I., Grozio, A., Lasiglie, D., Basile, G., Sturla, L., Magnone, M., et al. (2012). The NAD+-dependent histone deacetylase SIRT6 promotes cytokine production and migration in pancreatic cancer cells by regulating Ca^{2+} responses. *J. Biol. Chem*. 287, 40924–40937. doi:10.1074/jbc.M112.405837

Bazhin, A. V., Yang, Y., D'Haese, J. G., Werner, J., Philippov, P. P., and Karakhanova, S. (2016). The novel mitochondria-targeted antioxidant SkQ1 modulates angiogenesis and inflammatory micromilieu in a murine orthotopic model of pancreatic cancer. *Int. J. Cancer*. 139, 130–139. doi:10.1002/ijc.30054

Becchetti, A., Crescioli, S., Zanieri, F., Petroni, G., Mercatelli, R., Coppola, S., et al. (2017). The conformational state of hERG1 channels determines integrin association, downstream signaling, and cancer progression. *Sci. Signal*. 10, eaaf3236. doi:10.1126/scisignal.aaf3236

Becchetti, A., Petroni, G., and Arcangeli, A. (2019). Ion channel conformations regulate integrin-dependent signaling. *Trends Cell Biol*. 29, 298–307. doi:10.1016/j.tcb.2018.12.005

Beceiro, S., Radin, J. N., Chatuvedi, R., Piazuelo, M. B., Horvarth, D. J., Cortado, H., et al. (2017). TRPM2 ion channels regulate macrophage polarization and gastric inflammation during Helicobacter pylori infection. *Mucosal Immunol*. 10, 493–507. doi:10.1038/mi.2016.60

Becker, A. E., Hernandez, Y. G., Frucht, H., and Lucas, A. L. (2014). Pancreatic ductal adenocarcinoma: risk factors, screening, and early detection. *World J. Gastroenterol*. 20, 11182–11198. doi:10.3748/wjg.v20.i32.11182

Beeton, C., and Chandy, K. G. (2005). Potassium channels, memory T-cells, and multiple sclerosis. *Neuroscientist*. 11, 550–562. doi:10.1177/1073858405278016

Benavides, J., Camelin, J. C., Mitrani, N., Flamand, F., Uzan, A., Legrand, J.-J., et al. (1985). 2-Amino-6-trifluoromethoxy benzothiazole, a possible antagonist of excitatory amino acid neurotransmission—II biochemical properties. *Neuropharmacology*. 24, 1085–1092. doi:10.1016/0028-3908(85)90196-0

Bianchi, G., Vuerich, M., Pellegatti, P., Marimpietri, D., Emionite, L., Marigo, I., et al. (2014). ATP/P2X7 axis modulates myeloid-derived suppressor cell functions in neuroblastoma microenvironment. *Cell Death Dis*. 5, e1135. doi:10.1038/cddis.2014.109

Biswas, S. K., and Mantovani, A. (2010). Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat. Immunol*. 11, 889–896. doi:10.1038/ni.1937

Bittner, S., Ruck, T., Fernández-Orth, J., and Meuth, S. G. (2014). TREK-king the blood-brain-barrier. *J. Neuroimmune Pharmacol*. 9, 293–301. doi:10.1007/s11481-014-9530-8

Blando, J., Sharma, A., Higa, M. G., Zhao, H., Vence, L., Yadav, S. S., et al. (2019). Comparison of immune infiltrates in melanoma and pancreatic cancer highlights VISTA as a potential target in pancreatic cancer. *Proc. Natl. Acad. Sci. U.S.A*. 116, 1692–1697. doi:10.1073/pnas.1811067116

Bonito, B. (2017). Studies of ion channel mechanisms in pancreatic ductal adenocarcinoma (PDAC). *Imp. Coll. Lond*. [Epub ahead of print]. doi:10.25560/45534

Bonito, B., Sauter, D. R. P., Schwab, A., Djamgoz, M. B. A., and Novak, I. (2016). KCa3.1 (Ik) modulates pancreatic cancer cell migration, invasion and proliferation: anomalous effects on TRAM-34. *Pflugers Arch. Eur. J. Physiol*. 468, 1865–1875. doi:10.1007/s00424-016-1891-9

Bortner, C. D., and Cidlowski, J. A. (2011). Life and death of lymphocytes: a volume regulation affair. *Cell. Physiol. Biochem 28*, 1079–1088. doi:10.1159/000335864

Bortner, C. D., and Cidlowski, J. A. (2014). Ion channels and apoptosis in cancer. *Philos. Trans. R. Soc. B Biol. Sci*. 369, 20130104. doi:10.1098/rstb.2013.0104

Brevet, M., Fucks, D., Chatelain, D., Regimbeau, J. M., Delcenserie, R., Sevestre, H., et al. (2009). Deregulation of 2 potassium channels in pancreas adenocarcinomas: implication of K_V1.3 gene promoter methylation. *Pancreas*. 38, 649–654. doi:10.1097/MPA.0b013e3181a56ebf

Brömmel, K., Maskri, S., Bulk, E., Pethő, Z., Rieke, M., Budde, T., et al. (2020a). Co-staining of K_{Ca}3.1 channels in NSCLC cells with a small-molecule fluorescent probe and antibody-based indirect immunofluorescence. *ChemMedChem*. [Epub ahead of print]. doi:10.1002/cmdc.202000652

Brömmel, K., Maskri, S., Maisuls, I., Konken, C. P., Rieke, M., Pethő, Z., et al. (2020b). Synthesis of small-molecule fluorescent probes for the in vitro imaging of calcium-activated potassium channel K_{Ca}3.1. *Angew. Chem. Int. Ed. Engl*. 59, 8277–8284. doi:10.1002/anie.202001201

Brunner, M., Maier, K., Rümmele, P., Jacobsen, A., Merkel, S., Benard, A., et al. (2020). Upregulation of CD20 positive B-cells and B-cell aggregates in the tumor infiltration zone is associated with better survival of patients with pancreatic ductal adenocarcinoma. *Int. J. Mol. Sci*. 21, 1179. doi:10.3390/ijms21051779

Bulk, E., Ay, A. S., Hammadi, M., Ouadid-Ahidouch, H., Schelhaas, S., Hascher, A., et al. (2015). Epigenetic dysregulation of KCa3.1 channels induces poor prognosis in lung cancer. *Int. J. Cancer*. 137, 1306–1317. doi:10.1002/ijc.29490

Bulk, E., Todesca, L. M., and Schwab, A. (2020). Ion channels in lung cancer. *Rev. Physiol. Biochem. Pharmacol*. [Epub ahead of print]. doi:10.1007/112_2020_29

Cahalan, M. D., and Chandy, K. G. (2009). The functional network of ion channels in T lymphocytes. *Immunol. Rev*. 231, 59–87. doi:10.1111/j.1600-065X.2009.00816.x

Carstens, J. L., De Sampaio, P. C., Yang, D., Barua, S., Wang, H., Rao, A., et al. (2017). Spatial computation of intratumoral T-cells correlates with survival of patients with pancreatic cancer. *Nat. Commun*. 8, 15095. doi:10.1038/ncomms15095

Castino, G. F., Cortese, N., Capretti, G., Serio, S., Di Caro, G., Mineri, R., et al. (2016). Spatial distribution of B cells predicts prognosis in human pancreatic

adenocarcinoma. *Oncoimmunology*. 5, e1085147. doi:10.1080/2162402X.2015.1085147

Cernuda, B., Fernandes, C. T., Allam, S. M., Orzillo, M., Suppa, G., Chang, Z. C., et al. (2019). The molecular determinants of R-roscovitine block of hERG channels. *PLoS One*. 14, e0217733. doi:10.1371/journal.pone.0217733

Chandy, K. G., and Norton, R. S. (2016). Immunology: channelling potassium to fight cancer. *Nature*. 537, 497–499. doi:10.1038/nature19467

Chauhan, A., Sun, Y., Sukumaran, P., Quenum Zangbede, F. O., Jondle, C. N., Sharma, A., et al. (2018). M1 macrophage polarization is dependent on TRPC1-mediated calcium entry. *iScience*. 8, 85–102. doi:10.1016/j.isci.2018.09.014

Cheah, B. C., Vucic, S., Krishnan, A., and Kiernan, M. C. (2010). Riluzole, neuroprotection and amyotrophic lateral sclerosis. *Curr. Med. Chem*. 17, 1942–1959. doi:10.2174/092986710791163939

Chen, L., Sampson, K. J., and Kass, R. S. (2016). Cardiac delayed rectifier potassium channels in health and disease. *Card. Electrophysiol. Clin*. 8, 307–322. doi:10.1016/j.ccep.2016.01.004

Chen, Q., Zhou, Y., Zhou, L., Fu, Z., Yang, C., Zhao, L., et al. (2020). TRPC6-dependent Ca^{2+} signaling mediates airway inflammation in response to oxidative stress via ERK pathway. *Cell Death Dis*. 11, 170. doi:10.1038/s41419-020-2360-0

Chimote, A. A., Balajthy, A., Arnold, M. J., Newton, H. S., Hajdu, P., Qualtieri, J., et al. (2018). A defect in KCa3.1 channel activity limits the ability of CD8+ T-cells from cancer patients to infiltrate an adenosine-rich microenvironment. *Sci. Signal*. 11, eaaq1616. doi:10.1126/scisignal.aaq1616

Chimote, A. A., Gawali, V. S., Newton, H. S., Wise-Draper, T. M., and Conforti, L. (2020). A compartmentalized reduction in membrane-proximal calmodulin reduces the immune surveillance capabilities of CD8+ T-cells in head and neck cancer. *Front. Pharmacol*. 11, 143. doi:10.3389/fphar.2020.00143

Chimote, A. A., Hajdu, P., Kucher, V., Boiko, N., Kuras, Z., Szilagyi, O., et al. (2013). Selective inhibition of KCa3.1 channels mediates adenosine regulation of the motility of human T-cells. *J. Immunol*. 191, 6273–6280. doi:10.4049/jimmunol.1300702

Clark, C. E., Hingorani, S. R., Mick, R., Combs, C., Tuveson, D. A., and Vonderheide, R. H. (2007). Dynamics of the immune reaction to pancreatic cancer from inception to invasion. *Cancer Res*. 67, 9518–9527. doi:10.1158/0008-5472.CAN-07-0175

Comes, N., Serrano-Albarrás, A., Capera, J., Serrano-Novillo, C., Condom, E., Ramón Y Cajal, S., et al. (2015). Involvement of potassium channels in the progression of cancer to a more malignant phenotype. *Biochim. Biophys. Acta – Biomembr*. 1848, 2477–2492. doi:10.1016/j.bbamem.2014.12.008

Cox, J. H., Hussell, S., Snødergaard, H., Roepstorff, K., Bui, J. V., Deer, J. R., et al. (2013). Antibody-mediated targeting of the Orai1 calcium channel inhibits T-cell function. *PLoS One*. 8, e82944. doi:10.1371/journal.pone.0082944

Crottès, D., Lin, Y. H. T., Peters, C. J., Gilchrist, J. M., Wiita, A. P., Jan, Y. N., et al. (2019). TMEM16A controls EGF-induced calcium signaling implicated in pancreatic cancer prognosis. *Proc. Natl. Acad. Sci. U.S.A*. 116, 13026–13035. doi:10.1073/pnas.1900703116

Cruse, G., Singh, S. R., Duffy, S. M., Doe, C., Saunders, R., Brightling, C. E., et al. (2011). Functional $K_{Ca}3.1$ K^+ channels are required for human fibrocyte migration. *J. Allergy Clin. Immunol*. 128, 1303–1309. doi:10.1016/j.jaci.2011.07.047.e2

Cruz-Monserrate, Z., Roland, C. L., Deng, D., Arumugam, T., Moshnikova, A., Andreev, O. A., et al. (2014). Targeting pancreatic ductal adenocarcinoma acidic microenvironment. *Sci. Rep*. 4, 4410. doi:10.1038/srep04410

Cucu, D., Chiritoiu, G., Petrescu, S., Babes, A., Stanica, L., Duda, D. G., et al. (2014). Characterization of functional transient receptor potential melastatin 8 channels in human pancreatic ductal adenocarcinoma cells. *Pancreas*. 43, 795–800. doi:10.1097/MPA.0000000000000106

Cvetkovic, L., Perisic, S., Titze, J., Jäck, H. M., and Schuh, W. (2019). The impact of hyperosmolality on activation and differentiation of B lymphoid cells. *Front. Immunol*. 10, 828. doi:10.3389/fimmu.2019.00828

Dauer, P., Zhao, X., Gupta, V. K., Sharma, N., Kesh, K., Gnamlin, P., et al. (2018). Inactivation of cancer-associated-fibroblasts disrupts oncogenic signaling in pancreatic cancer cells and promotes its regression. *Canc. Res*. 78, 1321–1333. doi:10.1158/0008-5472.CAN-17-2320

De Marchi, E., Orioli, E., Pegoraro, A., Sangaletti, S., Portararo, P., Curti, A., et al. (2019). The P2X7 receptor modulates immune cells infiltration, ectonucleotidases expression and extracellular ATP levels in the tumor microenvironment. *Oncogene*. 38, 3636–3650. doi:10.1038/s41388-019-0684-y

De Marchi, U., Sassi, N., Fioretti, B., Catacuzzeno, L., Cereghetti, G. M., Szabò, I., et al. (2009). Intermediate conductance Ca^{2+}-activated potassium channel (KCa3.1) in the inner mitochondrial membrane of human colon cancer cells. *Cell Calcium*. 45, 509–516. doi:10.1016/j.ceca.2009.03.014

De Monte, L., Reni, M., Tassi, E., Clavenna, D., Papa, I., Recalde, H., et al. (2011). Intratumor T helper type 2 cell infiltrate correlates with cancer-associated fibroblast thymic stromal lymphopoietin production and reduced survival in pancreatic cancer. *J. Exp. Med*. 208, 469–478. doi:10.1084/jem.20101876

Debono, M. W., Le Guern, J., Canton, T., Doble, A., and Pradier, L. (1993). Inhibition by riluzole of electrophysiological responses mediated by rat kainate and NMDA receptors expressed in Xenopus oocytes. *Eur. J. Pharmacol*. 235, 283–289. doi:10.1016/0014-2999(93)90147-A

DeCoursey, T. E., Morgan, D., Musset, B., and Cherny, V. V (2016). Insights into the structure and function of H_V1 from a meta-analysis of mutation studies. *J. Gen. Physiol*. 148, 97–118. doi:10.1085/jgp.201611619

Deicher, A., Andersson, R., Tingstedt, B., Lindell, G., Bauden, M., and Ansari, D. (2018). Targeting dendritic cells in pancreatic ductal adenocarcinoma. *Canc. Cell Int*. 18, 85. doi:10.1186/s12935-018-0585-0

Delierneux, C., Kouba, S., Shanmughapriya, S., Potier-Cartereau, M., Trebak, M., and Hempel, N. (2020). Mitochondrial calcium regulation of redox signaling in cancer. *Cells*. 9, 432. doi:10.3390/cells9020432

Desai, B. N., and Leitinger, N. (2014). Purinergic and calcium signaling in macrophage function and plasticity. *Front. Immunol*. 5, 580. doi:10.3389/fimmu.2014.00580

Deutsch, C., and Lee, S. C. (1989). Modulation of K+ currents in human lymphocytes by pH. *J. Physiol*. 413, 399–413. doi:10.1113/jphysiol.1989.sp017660

Devor, D. C., Singh, A. K., Frizzell, R. A., and Bridges, R. J. (1996). Modulation of Cl^- secretion by benzimidazolones. I. Direct activation of a Ca^{2+}-dependent K^+ channel. *Am. J. Physiol*. 271, L775–L784. doi:10.1152/ajplung.1996.271.5.l775

Dhennin-Duthille, I., Gautier, M., Faouzi, M., Guilbert, A., Brevet, M., Vaudry, D., et al. (2011). High expression of transient receptor potential channels in human breast cancer epithelial cells and tissues: correlation with pathological parameters. *Cell. Physiol. Biochem*. 28, 813–822. doi:10.1159/000335795

Di, A., Xiong, S., Ye, Z., Malireddi, R. K. S., Kometani, S., Zhong, M., et al. (2018). The TWIK2 potassium efflux channel in macrophages mediates NLRP3 inflammasome-induced inflammation. *Immunity*. 49, 56–65.e4. doi:10.1016/j.immuni.2018.04.032

Di Caro, G., Cortese, N., Castino, G. F., Grizzi, F., Gavazzi, F., Ridolfi, C., et al. (2015). Dual prognostic significance of tumour-Associated macrophages in human pancreatic adenocarcinoma treated or untreated with chemotherapy. *Gut*. 65, 1710–1720. doi:10.1136/gutjnl-2015-309193

Di, L., Srivastava, S., Zhdanova, O., Ding, Y., Li, Z., Wulff, H., et al. (2010). Inhibition of the K^+ channel KCa3.1 ameliorates T cell-mediated colitis. *Proc. Natl. Acad. Sci. U.S.A*. 107, 1541–1546. doi:10.1073/pnas.0910133107

Ding, X. W., Luo, H. S., Jin, X., Yan, J. J., and Ai, Y. W. (2007). Aberrant expression of Eag1 potassium channels in gastric cancer patients and cell lines. *Med. Oncol*. 24, 345–350. doi:10.1007/s12032-007-0015-y

Djamgoz, M. B. A., and Onkal, R. (2012). Persistent current blockers of voltage-gated sodium channels: a clinical opportunity for controlling metastatic disease. *Recent Pat. Anti-Canc. Drug Discov*. 8, 66–84. doi:10.2174/15748928130107

Djamgoz, M. B. A., Coombes, R. C., and Schwab, A. (2014). Ion transport and cancer: from initiation to metastasis. *Philos. Trans. R. Soc. Lond. B. Biol. Sci*. 369, 20130092. doi:10.1098/rstb.2013.0092

Djamgoz, M. B. A., Fraser, S. P., and Brackenbury, W. J. (2019). In vivo evidence for voltage-gated sodium channel expression in carcinomas and potentiation of metastasis. *Cancers*. 11, 1675. doi:10.3390/cancers11111675

Dong, H., Shim, K. N., Li, J. M. J., Estrema, C., Ornelas, T. A., Nguyen, F., et al. (2010). Molecular mechanisms underlying Ca^{2+}-mediated motility of human pancreatic duct cells. *Am. J. Physiol. – Cell Physiol*. 299, 1493–1503. doi:10.1152/ajpcell.00242.2010

Dovmark, T. H., Saccomano, M., Hulikova, A., Alves, F., and Swietach, P. (2017). Connexin-43 channels are a pathway for discharging lactate from glycolytic pancreatic ductal adenocarcinoma cells. *Oncogene*. 36, 4538–4550. doi:10.1038/onc.2017.71

Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J., and Schreiber, R. D. (2002). Cancer immunoediting: from immunosurveillance to tumor escape. *Nat. Immunol.* 3, 991–998. doi:10.1038/ni1102-991

Duranti, C., and Arcangeli, A. (2019). *Ion channel targeting with antibodies and antibody fragments for cancer diagnosis*. Basel, Switzerland: Antibodies, Vol. 8. doi:10.3390/antib8020033

Duranti, C., Carraresi, L., Sette, A., Stefanini, M., Lottini, T., Crescioli, S., et al. (2018). Generation and characterization of novel recombinant anti-hERG1 scFv antibodies for cancer molecular imaging. *Oncotarget*. 9, 34972–34989. doi:10.18632/oncotarget.26200

Eil, R., Vodnala, S. K., Clever, D., Klebanoff, C. A., Sukumar, M., Pan, J. H., et al. (2016). Ionic immune suppression within the tumour microenvironment limits T-cell effector function. *Nature*. 537, 539–543. doi:10.1038/nature19364

El Chemaly, A., Nunes, P., Jimaja, W., Castelbou, C., and Demaurex, N. (2014). Hv1 proton channels differentially regulate the pH of neutrophil and macrophage phagosomes by sustaining the production of phagosomal ROS that inhibit the delivery of vacuolar ATPases. *J. Leukoc. Biol.* 95, 827–839. doi:10.1189/jlb.0513251

El Chemaly, A., Okochi, Y., Sasaki, M., Arnaudeau, S., Okamura, Y., and Demaurex, N. (2010). VSOP/Hv1 proton channels sustain calcium entry, neutrophil migration, and superoxide production by limiting cell depolarization and acidification. *J. Exp. Med.* 207, 129–139. doi:10.1084/jem.20091837

Estes, D. J., Memarsadeghi, S., Lundy, S. K., Marti, F., Mikol, D. D., Fox, D. A., et al. (2008). High-throughput profiling of ion channel activity in primary human lymphocytes. *Anal. Chem.* 80, 3728–3735. doi:10.1021/ac800164v

Ezhilarasan, D., Sokal, E., and Najimi, M. (2018). Hepatic fibrosis: it is time to go with hepatic stellate cell-specific therapeutic targets. *Hepatobiliary Pancreat. Dis. Int.* 17, 192–197. doi:10.1016/j.hbpd.2018.04.003

Fanger, C. M., Neben, A. L., and Cahalan, M. D. (2000). Differential Ca 2+ influx, K Ca channel activity, and Ca^{2+} clearance distinguish Th1 and Th2 lymphocytes. *J. Immunol.* 164, 1153–1160. doi:10.4049/jimmunol.164.3.1153

Faouzi, M., Hague, F., Geerts, D., Ay, A. S., Potier-Cartereau, M., Ahidouch, A., et al. (2016). Functional cooperation between KCa3.1 and TRPC1 channels in human breast cancer: role in cell proliferation and patient prognosis. *Oncotarget*. 7, 36419–36435. doi:10.18632/oncotarget.9261

Farfariello, V., Prevarskaya, N., and Gkika, D. (2020). ion channel profiling in prostate cancer: toward cell population-specific screening. *Rev. Physiol. Biochem. Pharmacol.* [Epub ahead of print]. doi:10.1007/112_2020_22

Farrell, A. S., Joly, M. M., Allen-Petersen, B. L., Worth, P. J., Lanciault, C., Sauer, D., et al. (2017). MYC regulates ductal-neuroendocrine lineage plasticity in pancreatic ductal adenocarcinoma associated with poor outcome and chemoresistance. *Nat. Commun.* 8, 1728. doi:10.1038/s41467-017-01967-6

Fecher-Trost, C., Wissenbach, U., and Weissgerber, P. (2017). TRPV6: from identification to function. *Cell Calcium*. 67, 116–122. doi:10.1016/j.ceca.2017.04.006

Fels, B., Bulk, E., Pethő, Z., and Schwab, A. (2018). The role of TRP channels in the metastatic cascade. *Pharmaceuticals*. 11, 48. doi:10.3390/ph11020048

Fels, B., Nielsen, N., and Schwab, A. (2016). Role of TRPC1 channels in pressure-mediated activation of murine pancreatic stellate cells. *Eur. Biophys. J.* 45, 657–670. doi:10.1007/s00249-016-1176-4

Feng, J., Yu, J., Pan, X., Li, Z., Chen, Z., Zhang, W., et al. (2014). HERG1 functions as an oncogene in pancreatic cancer and is downregulated by miR-96. *Oncotarget*. 5, 5832–5844. doi:10.18632/oncotarget.2200

Feng, L. L., Cai, Y. Q., Zhu, M. C., Xing, L. J., and Wang, X. (2020). The yin and yang functions of extracellular ATP and adenosine in tumor immunity. *Canc. Cell Int.* 20, 110. doi:10.1186/s12935-020-01195-x

Ferdek, P. E., and Jakubowska, M. A. (2017). Biology of pancreatic stellate cells—more than just pancreatic cancer. *Pflugers Arch. Eur. J. Physiol.* 469, 1039–1050. doi:10.1007/s00424-017-1968-0

Fernández, A., Pupo, A., Mena-Ulecia, K., and Gonzalez, C. (2016). Pharmacological modulation of proton channel hv1 in cancer therapy: future perspectives. *Mol. Pharmacol.* 90, 385–402. doi:10.1124/mol.116.103804

Ferreri, A. J. M., Cecchetti, C., Kiesewetter, B., Sassone, M., Calimeri, T., Perrone, S., et al. (2018). Clarithromycin as a 'repurposing drug' against MALT lymphoma. *Br. J. Haematol.* 182, 913–915. doi:10.1111/bjh.14878

Feske, S., Skolnik, E. Y., and Prakriya, M. (2012). Ion channels and transporters in lymphocyte function and immunity. *Nat. Rev. Immunol.* 12, 532–547. doi:10.1038/nri3233

Feske, S., Wulff, H., and Skolnik, E. Y. (2015). Ion channels in innate and adaptive immunity. *Ann. Rev. Immunol.* 33, 291–353. doi:10.1146/annurev-immunol-032414-112212

Freise, C., Heldwein, S., Erben, U., Hoyer, J., Köhler, R., Jöhrens, K., et al. (2015). K+-channel inhibition reduces portal perfusion pressure in fibrotic rats and fibrosis associated characteristics of hepatic stellate cells. *Liver Int.* 35, 1244–1252. doi:10.1111/liv.12681

Fu, S., Hirte, H., Welch, S., Ilenchuk, T. T., Lutes, T., Rice, C., et al. (2017). First-in-human phase I study of SOR-C13, a TRPV6 calcium channel inhibitor, in patients with advanced solid tumors. *Invest. New Drugs*. 35, 324–333. doi:10.1007/s10637-017-0438-z

Fu, Y., Liu, S., Zeng, S., and Shen, H. (2018). The critical roles of activated stellate cells-mediated paracrine signaling, metabolism and onco-immunology in pancreatic ductal adenocarcinoma. *Mol. Cancer*. 17, 62. doi:10.1186/s12943-018-0815-z

Gabrilovich, D. I., Ostrand-Rosenberg, S., and Bronte, V. (2012). Coordinated regulation of myeloid cells by tumours. *Nat. Rev. Immunol.* 12, 253–268. doi:10.1038/nri3175

Galon, J., and Bruni, D. (2019). Approaches to treat immune hot, altered and cold tumours with combination immunotherapies. *Nat. Rev. Drug Discov.* 18, 197–218. doi:10.1038/s41573-018-0007-y

Galon, J., Mlecnik, B., Bindea, G., Angell, H. K., Berger, A., Lagorce, C., et al. (2014). Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. *J. Pathol.* 232, 199–209. doi:10.1002/path.4287

Gao, Y., Hanley, P. J., Rinné, S., Zuzarte, M., and Daut, J. (2010). Calcium-activated K^+ channel ($K_{Ca}3.1$) activity during Ca^{2+} store depletion and store-operated Ca(2+) entry in human macrophages. *Cell Calcium*. 48, 19–27. doi:10.1016/j.ceca.2010.06.002

García-Ferreiro, R. E., Kerschensteiner, D., Major, F., Monje, F., Stühmer, W., and Pardo, L. A. (2004). Mechanism of block of hEag1 K^+ channels by imipramine and astemizole. *J. Gen. Physiol.* 124, 301–317. doi:10.1085/jgp.200409041

Garofalo, S., Cocozza, G., Porzia, A., Inghilleri, M., Raspa, M., Scavizzi, F., et al. (2020). Natural killer cells modulate motor neuron-immune cell cross talk in models of Amyotrophic Lateral Sclerosis. *Nat. Commun.* 11, 1773. doi:10.1038/s41467-020-15644-8

Garrelts, J. C. (1991). Clofazimine: a review of its use in leprosy and Mycobacterium avium complex infection. *DICP*. 25, 525–531. doi:10.1177/106002809102500513

Gavrilova-Ruch, O., Schonherr, K., Gessner, G., Schonherr, R., Klapperstuck, T., Wohlrab, W., et al. (2002). Effects of imipramine on ion channels and proliferation of IGR1 melanoma cells\rCardiac complications of radiation therapy. *J. Membr. Biol.* 188, 137–149. doi:10.1007/s00232-001-0181-3

Gershkovitz, M., Caspi, Y., Fainsod-Levi, T., Katz, B., Michaeli, J., Khawaled, S., et al. (2018). TRPM2 mediates neutrophil killing of disseminated tumor cells. *Cancer Res.* 78, 2680–2690. doi:10.1158/0008-5472.CAN-17-3614

Ghanshani, S., Wulff, H., Miller, M. J., Rohm, H., Neben, A., Gutman, G. A., et al. (2000). Up-regulation of the IKCa1 potassium channel during T-cell activation: molecular mechanism and functional consequences. *J. Biol. Chem.* 275, 37137–37149. doi:10.1074/jbc.M003941200

Giannuzzo, A., Pedersen, S. F., and Novak, I. (2015). The P2X7 receptor regulates cell survival, migration and invasion of pancreatic ductal adenocarcinoma cells. *Mol. Canc.* 14, 203. doi:10.1186/s12943-015-0472-4

Giannuzzo, A., Saccomano, M., Napp, J., Ellegaard, M., Alves, F., and Novak, I. (2016). Targeting of the P2X7 receptor in pancreatic cancer and stellate cells. *Int. J. Canc.* 139, 2540–2552. doi:10.1002/ijc.30380

Gillies, R. J., Raghunand, N., Karczmar, G. S., and Bhujwalla, Z. M. (2002). MRI of the tumor microenvironment. *J. Magn. Reson. Imag.* 16, 430–450. doi:10.1002/jmri.10181

Girault, A., Ahidouch, A., and Ouadid-Ahidouch, H. (2020). Roles for Ca2+ and K+ channels in cancer cells exposed to the hypoxic tumour microenvironment. *Biochim. Biophys. Acta – Mol. Cell Res.* 1867, 118644. doi:10.1016/j.bbamcr.2020.118644

Gnerlich, J. L., Mitchem, J. B., Weir, J. S., Sankpal, N. V., Kashiwagi, H., Belt, B. A., et al. (2010). Induction of Th17 cells in the tumor microenvironment improves

survival in a murine model of pancreatic cancer. *J. Immunol.* 185, 4063–4071. doi:10.4049/jimmunol.0902609

Gocke, A. R., Lebson, L. A., Grishkan, I. V., Hu, L., Nguyen, H. M., Whartenby, K. A., et al. (2012). Kv1.3 deletion biases T-cells toward an immunoregulatory phenotype and renders mice resistant to autoimmune Encephalomyelitis. *J. Immunol.* 188, 5877–5886. doi:10.4049/jimmunol.1103095

Gómez-Varela, D., Zwick-Wallasch, E., Knötgen, H., Sánchez, A., Hettmann, T., Ossipov, D., et al. (2007). Monoclonal antibody blockade of the human Eag1 potassium channel function exerts antitumor activity. *Cancer Res.* 67, 7343–7349. doi:10.1158/0008-5472.CAN-07-0107

Gürlevik, E., Fleischmann-Mundt, B., Brooks, J., Demir, I. E., Steiger, K., Ribback, S., et al. (2016). Administration of gemcitabine after pancreatic tumor resection in mice induces an antitumor immune response mediated by natural killer cells. *Gastroenterology.* 151, 338–350. doi:10.1053/j.gastro.2016.05.004.e7

Gwack, Y., Srikanth, S., Oh-hora, M., Hogan, P. G., Lamperti, E. D., Yamashita, M., et al. (2008). Hair loss and defective T- and B-cell function in mice lacking ORAI1. *Mol. Cell. Biol.* 28, 5209–5222. doi:10.1128/mcb.00360-08

Haanes, K. A., Schwab, A., and Novak, I. (2012). The P2X7 receptor supports both life and death in fibrogenic pancreatic stellate cells. *PLoS One.* 7, e51164. doi:10.1371/journal.pone.0051164

Habtezion, A., Edderkaoui, M., and Pandol, S. J. (2016). Macrophages and pancreatic ductal adenocarcinoma. *Cancer Lett.* 381, 211–216. doi:10.1016/j.canlet.2015.11.049

Haeberle, L., Steiger, K., Schlitter, A. M., Safi, S. A., Knoefel, W. T., Erkan, M., et al. (2018). Stromal heterogeneity in pancreatic cancer and chronic pancreatitis. *Pancreatology.* 18, 536–549. doi:10.1016/j.pan.2018.05.004

Han, Y., Shewan, A. M., and Thorn, P. (2016). HCO3- transport through anoctamin/transmembrane protein ANO1/TMEM16A in pancreatic acinar cells regulates luminal pH. *J. Biol. Chem.* 291, 20345–20352. doi:10.1074/jbc.M116.750224

Hartel, M., Di Mola, F. F., Selvaggi, F., Mascetta, G., Wente, M. N., Felix, K., et al. (2006). Vanilloids in pancreatic cancer: potential for chemotherapy and pain management. *Gut.* 55, 519–528. doi:10.1136/gut.2005.073205

Hartung, F., Krüwel, T., Shi, X., Pfizenmaier, K., Kontermann, R., Chames, P., et al. (2020). A novel anti-Kv10.1 nanobody fused to single-chain TRAIL enhances apoptosis induction in cancer cells. *Front. Pharmacol.* 11, 686. doi:10.3389/fphar.2020.00686

Harvey, J. B., Phan, L. H., Villarreal, O. E., and Bowser, J. L. (2020). CD73's potential as an immunotherapy target in gastrointestinal cancers. *Front. Immunol.* 11, 16–18. doi:10.3389/fimmu.2020.00508

Hashim, A. I., Zhang, X., Wojtkowiak, J. W., Martinez, G. V., and Gillies, R. J. (2011). Imaging pH and metastasis. *NMR Biomed.* 24, 582–591. doi:10.1002/nbm.1644.Imaging

Hayashi, M., and Novak, I. (2013). Molecular basis of potassium channels in pancreatic duct epithelial cells. *Channels.* 7, 432–441. doi:10.4161/chan.26100

Hayashi, M., Wang, J., Hede, S. E., and Novak, I. (2012). An intermediate-conductance Ca^{2+}-activated K^+ channel is important for secretion in pancreatic duct cells. *Am. J. Physiol. Cell Physiol.* 303, C151–C159. doi:10.1152/ajpcell.00089.2012

He, W., Xu, J., Mu, R., Li, Q., Lv, D. lun., Huang, Z., et al. (2020). High-salt diet inhibits tumour growth in mice via regulating myeloid-derived suppressor cell differentiation. *Nat. Commun.* 11, 1–17. doi:10.1038/s41467-020-15524-1

Hegde, S., Krisnawan, V. E., Herzog, B. H., Zuo, C., Breden, M. A., Knolhoff, B. L., et al. (2020). Dendritic cell paucity leads to dysfunctional immune surveillance in pancreatic cancer. *Canc. Cell.* 37, 289–307.e9. doi:10.1016/j.ccell.2020.02.008

Hegde, V. L., Nagarkatti, P. S., and Nagarkatti, M. (2011). Role of myeloid-derived suppressor cells in amelioration of experimental autoimmune hepatitis following activation of TRPV1 receptors by cannabidiol. *PLoS One.* 6, e18281. doi:10.1371/journal.pone.0018281

Hemmerlein, B., Weseloh, R. M., de Queiroz, F. M., Knötgen, H., Sánchez, A., Rubio, M. E., et al. (2006). Overexpression of Eag1 potassium channels in clinical tumours. *Mol. Canc.* 5, 41. doi:10.1186/1476-4598-5-41

Henke, E., Nandigama, R., and Ergün, S. (2020). Extracellular matrix in the tumor microenvironment and its impact on cancer therapy. *Front. Mol. Biosci.* 6, 1–24. doi:10.3389/fmolb.2019.00160

Henríquez, C., Riquelme, T. T., Vera, D., Julio-Kalajzić, F., Ehrenfeld, P., Melvin, J. E., et al. (2016). The calcium-activated potassium channel KCa3.1 plays a central role in the chemotactic response of mammalian neutrophils. *Acta Physiol.* 216, 132–145. doi:10.1111/apha.12548

Hessmann, E., Buchholz, S. M., Demir, I. E., Singh, S. K., Gress, T. M., Ellenrieder, V., et al. (2020). Microenvironmental determinants of pancreatic cancer. *Physiol. Rev.* 100, 1707–1751. doi:10.1152/physrev.00042.2019

High, R. A., Randtke, E. A., Jones, K. M., Lindeman, L. R., Ma, J. C., Zhang, S., et al. (2019). Extracellular acidosis differentiates pancreatitis and pancreatic cancer in mouse models using acidoCEST MRI. *Neoplasia.* 21, 1085–1090. doi:10.1016/j.neo.2019.09.004

Hiraoka, N., Ino, Y., Sekine, S., Tsuda, H., Shimada, K., Kosuge, T., et al. (2010). Tumour necrosis is a postoperative prognostic marker for pancreatic cancer patients with a high interobserver reproducibility in histological evaluation. *Br. J. Cancer.* 103, 1057–1065. doi:10.1038/sj.bjc.6605854

Hoffmann, E. K., Lambert, I. H., and Pedersen, S. F. (2009). Physiology of cell volume regulation in vertebrates. *Physiol. Rev.* 89, 193–277. doi:10.1152/physrev.00037.2007

Honoré, E. (2007). The neuronal background K2P channels: focus on TREK1. *Nat. Rev. Neurosci.* 8, 251–261. doi:10.1038/nrn2117

Hoonjan, M., Jadhav, V., and Bhatt, P. (2018). Arsenic trioxide: insights into its evolution to an anticancer agent. *J. Biol. Inorg. Chem. JBIC a Publ. Soc. Biol. Inorg. Chem.* 23, 313–329. doi:10.1007/s00775-018-1537-9

Hu, H., Hang, J. J., Han, T., Zhuo, M., Jiao, F., and Wang, L. W. (2016). The M2 phenotype of tumor-associated macrophages in the stroma confers a poor prognosis in pancreatic cancer. *Tumor Biol.* 37, 8657–8664. doi:10.1007/s13277-015-4741-z

Hu, H., Jiao, F., Han, T., and Wang, L.-W. (2015). Functional significance of macrophages in pancreatic cancer biology. *Tumour Biol. J. Int. Soc. Oncodevelopmental Biol. Med.* 36, 9119–9126. doi:10.1007/s13277-015-4127-2

Hu, L. P., Zhang, X. X., Jiang, S. H., Tao, L. Y., Li, Q., Zhu, L. L., et al. (2019a). Targeting purinergic receptor P2Y2 prevents the growth of pancreatic ductal adenocarcinoma by inhibiting cancer cell glycolysis. *Clin. Canc. Res.* 25, 1318–1330. doi:10.1158/1078-0432.CCR-18-2297

Hu, S., Yang, J., Shangguan, J., Eresen, A., Li, Y., Ma, Q., et al. (2019b). Natural killer cell-based adoptive transfer immunotherapy for pancreatic ductal adenocarcinoma in a $Kras^{LSL\text{-}G12D}p53^{LSL\text{-}R172HP}$dx1-Cre mouse model. *Am. J. Cancer Res.* 9, 1757–1765.

Huang, X., and Jan, L. Y. (2014). Targeting potassium channels in cancer. *J. Cell. Biol.* 206, 151–162. doi:10.1083/jcb.201404136

Ijichi, H., Chytil, A., Gorska, A. E., Aakre, M. E., Bierie, B., Tada, M., et al. (2011). Inhibiting Cxcr2 disrupts tumor-stromal interactions and improves survival in a mouse model of pancreatic ductal adenocarcinoma. *J. Clin. Invest.* 121, 4106–4117. doi:10.1172/JCI42754

Ivanova, E. A., and Orekhov, A. N. (2015). T Helper lymphocyte subsets and plasticity in autoimmunity and cancer: an overview. *Biomed Res. Int.* 2015, 327470. doi:10.1155/2015/327470.2015

Jäger, H., Dreker, T., Buck, A., Giehl, K., Gress, T., and Grissmer, S. (2004). Blockage of intermediate-conductance Ca^{2+}-activated K^+ channels inhibit human pancreatic cancer cell growth *in Vitro*. *Mol. Pharmacol.* 65, 630–638. doi:10.1124/mol.65.3.630

Jiang, S.-H., Zhu, L. L., Zhang, M., Li, R. K., Yang, Q., Yan, J. Y., et al. (2019). GABRP regulates chemokine signalling, macrophage recruitment and tumour progression in pancreatic cancer through tuning KCNN4-mediated Ca2+ signalling in a GABA-independent manner. *Gut.* 68, 1994–2006. doi:10.1136/gutjnl-2018-317479

Jiang, S., Zhu, L., Yang, J., Hu, L., Gu, J., Xing, X., et al. (2017). Integrated expression profiling of potassium channels identifys KCNN4 as a prognostic biomarker of pancreatic cancer. *Biochem. Biophys. Res. Commun.* 494, 113–119. doi:10.1016/j.bbrc.2017.10.072

Jin, S., Chin, J., Kitson, C., Woods, J., Majmudar, R., Carvajal, V., et al. (2013). Natural regulatory T-cells are resistant to calcium release-activated calcium (CRAC/ORAI) channel inhibition. *Int. Immunol.* 25, 497–506. doi:10.1093/intimm/dxt013

Kale, V. P., Amin, S. G., and Pandey, M. K. (2015). Targeting ion channels for cancer therapy by repurposing the approved drugs. *Biochim. Biophys. Acta.* 1848, 2747–2755. doi:10.1016/j.bbamem.2015.03.034

Karmakar, M., Katsnelson, M. A., Dubyak, G. R., and Pearlman, E. (2016). Neutrophil P2X7 receptors mediate NLRP3 inflammasome-dependent IL-1β secretion in response to ATP. *Nat. Commun.* 7, 10555. doi:10.1038/ncomms10555

Kindzelskii, A. L., and Petty, H. R. (2005). Ion channel clustering enhances weak electric field detection by neutrophils: apparent roles of SKF96365-sensitive cation channels and myeloperoxidase trafficking in cellular responses. *Eur. Biophys. J.* 35, 1–26. doi:10.1007/s00249-005-0001-2

Kis-Toth, K., Hajdu, P., Bacskai, I., Szilagyi, O., Papp, F., Szanto, A., et al. (2011). Voltage-gated sodium channel Nav1.7 maintains the membrane potential and regulates the activation and chemokine-induced migration of a monocyte-derived dendritic cell subset. *J. Immunol.* 187, 1273–1280. doi:10.4049/jimmunol.1003345

Klebanoff, C. A., Gattinoni, L., Torabi-Parizi, P., Kerstann, K., Cardones, A. R., Finkelstein, S. E., et al. (2005). Central memory self/tumor-reactive CD^{8+} T-cells confer superior antitumor immunity compared with effector memory T-cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 9571–9576. doi:10.1073/pnas.0503726102

Klebanoff, C. A., Scott, C. D., Leonardi, A. J., Yamamoto, T. N., Cruz, A. C., Ouyang, C., et al. (2016). Memory T cell-driven differentiation of naive cells impairs adoptive immunotherapy. *J. Clin. Invest.* 126, 318–334. doi:10.1172/JCI81217

Klumpp, L., Sezgin, E. C., Skardelly, M., Eckert, F., and Huber, S. M. (2018). KCa3.1 channels and glioblastoma: in vitro studies. *Curr. Neuropharmacol.* 16, 627–635. doi:10.2174/1570159X15666170808115821

Knudsen, E. S., Vail, P., Balaji, U., Ngo, H., Botros, I. W., Makarov, V., et al. (2017). Stratification of pancreatic ductal adenocarcinoma: combinatorial genetic, stromal, and immunologic markers. *Clin. Cancer Res.* 23, 4429–4440. doi:10.1158/1078-0432.CCR-17-0162

Kondratska, K., Kondratskyi, A., Yassine, M., Lemonnier, L., Lepage, G., Morabito, A., et al. (2014). Orai1 and STIM1 mediate SOCE and contribute to apoptotic resistance of pancreatic adenocarcinoma. *Biochim. Biophys. Acta – Mol. Cell Res.* 1843, 2263–2269. doi:10.1016/j.bbamcr.2014.02.012

Koshy, S., Wu, D., Hu, X., Tajhya, R. B., Huq, R., Khan, F. S., et al. (2013). Blocking KCa3.1 channels increases tumor cell killing by a subpopulation of human natural killer lymphocytes. *PLoS One.* 8, 1–11. doi:10.1371/journal.pone.0076740

Kovalenko, I., Glasauer, A., Schöckel, L., Sauter, D. R. P. P., Ehrmann, A., Sohler, F., et al. (2016). Identification of $K_{Ca}3.1$ channel as a novel regulator of oxidative phosphorylation in a subset of pancreatic carcinoma cell lines. *PLoS One.* 11, 1–20. doi:10.1371/journal.pone.0160658

Krause, K. H., and Welsh, M. J. (1990). Voltage-dependent and Ca2+-activated ion channels in human neutrophils. *J. Clin. Invest.* 85, 491–498. doi:10.1172/JCI114464

Kuntze, A., Goetsch, O., Fels, B., Najder, K., Unger, A., Wilhelmi, M., et al. (2020). Protonation of Piezo1 impairs cell-matrix interactions of pancreatic stellate cells. *Front. Physiol.* 11, 1–15. doi:10.3389/fphys.2020.00089

Künzli, B. M., Berberat, P. O., Giese, T., Csizmadia, E., Kaczmarek, E., Baker, C., et al. (2007). Upregulation of CD39/NTPDases and P2 receptors in human pancreatic disease. *Am. J. Physiol. - Gastrointest. Liver Physiol.* 292, 223–231. doi:10.1152/ajpgi.00259.2006

Kurahara, H., Shinchi, H., Mataki, Y., Maemura, K., Noma, H., Kubo, F., et al. (2011). Significance of M2-polarized tumor-associated macrophage in pancreatic cancer. *J. Surg. Res.* 167, e211-9. doi:10.1016/j.jss.2009.05.026

Lachowski, D., Cortes, E., Pink, D., Chronopoulos, A., Karim, S. A., Morton, J. P., et al. (2017). Substrate rigidity controls activation and durotaxis in pancreatic stellate cells. *Sci. Rep.* 7, 1–12. doi:10.1038/s41598-017-02689-x

Lamanauskas, N., and Nistri, A. (2008). Riluzole blocks persistent Na+ and Ca2+ currents and modulates release of glutamate via presynaptic NMDA receptors on neonatal rat hypoglossal motoneurons *in vitro*. *Eur. J. Neurosci.* 27, 2501–2514. doi:10.1111/j.1460-9568.2008.06211.x

Lastraioli, E., Guasti, L., Crociani, O., Polvani, S., Hofmann, G., Witchel, H., et al. (2004). herg1 gene and HERG1 protein are overexpressed in colorectal cancers and regulate cell invasion of tumor cells. *Cancer Res.* 64, 606–611. doi:10.1158/0008-5472.CAN-03-2360

Lastraioli, E., Perrone, G., Sette, A., Fiore, A., Crociani, O., Manoli, S., et al. (2015). HERG1 channels drive tumour malignancy and may serve as prognostic factor in pancreatic ductal adenocarcinoma. *Br. J. Canc.* 112, 1076–1087. doi:10.1038/bjc.2015.28

Lauritzen, I., Chemin, J., Honoré, E., Jodar, M., Guy, N., Lazdunski, M., et al. (2005). Cross-talk between the mechano-gated K2P channel TREK-1 and the actin cytoskeleton. *EMBO Rep.* 6, 642–648. doi:10.1038/sj.embor.7400449

Leanza, L., Romio, M., Becker, K. A., Azzolini, M., Trentin, L., Managò, A., et al. (2017). Direct pharmacological targeting of a mitochondrial ion channel selectively Kills tumor cells in vivo. *Canc. Cell.* 31, 516–531.e10. doi:10.1016/j.ccell.2017.03.003

Leppkes, M., Maueröder, C., Hirth, S., Nowecki, S., Günther, C., Billmeier, U., et al. (2016). Externalized decondensed neutrophil chromatin occludes pancreatic ducts and drives pancreatitis. *Nat. Commun.* 7, 10973. doi:10.1038/ncomms10973

Leslie, T. K., James, A. D., Zaccagna, F., Grist, J. T., Deen, S., Kennerley, A., et al. (2019). Sodium homeostasis in the tumour microenvironment. *Biochim. Biophys. Acta – Rev. Cancer.* 1872, 188304. doi:10.1016/j.bbcan.2019.07.001

Li, S. Q., Su, N., Gong, P., Zhang, H. B., Liu, J., Wang, D., et al. (2017). The expression of formyl peptide receptor 1 is correlated with tumor invasion of human colorectal cancer. *Sci. Rep.* 7, 5918. doi:10.1038/s41598-017-06368-9

Lin, B. L., Matera, D., Doerner, J. F., Zheng, N., Del Camino, D., Mishra, S., et al. (2019). *In vivo* selective inhibition of TRPC6 by antagonist BI 749327 ameliorates fibrosis and dysfunction in cardiac and renal disease. *Proc. Natl. Acad. Sci. U.S.A.* 116, 10156–10161. doi:10.1073/pnas.1815354116

Lin, R., Wang, Y., Chen, Q., Liu, Z., Xiao, S., Wang, B., et al. (2018). TRPM2 promotes the proliferation and invasion of pancreatic ductal adenocarcinoma. *Mol. Med. Rep.* 17, 7537–7544. doi:10.3892/mmr.2018.8816

Lindemann, O., Rossaint, J., Najder, K., Schimmelpfennig, S., Hofschröer, V., Wälte, M., et al. (2020). Intravascular adhesion and recruitment of neutrophils in response to CXCL1 depends on their TRPC6 channels. *J. Mol. Med.* 98, 349–360. doi:10.1007/s00109-020-01872-4

Lindemann, O., Strodthoff, C., Horstmann, M., Nielsen, N., Jung, F., Schimmelpfennig, S., et al. (2015). TRPC1 regulates fMLP-stimulated migration and chemotaxis of neutrophil granulocytes. *Biochim. Biophys. Acta.* 1853, 2122–2130. doi:10.1016/j.bbamcr.2014.12.037

Lindemann, O., Umlauf, D., Frank, S., Schimmelpfennig, S., Bertrand, J., Pap, T., et al. (2013). TRPC6 regulates CXCR2-mediated chemotaxis of murine neutrophils. *J. Immunol.* 190, 5496–5505. doi:10.4049/jimmunol.1201502

Ling, Q., and Kalthoff, H. (2020). Transportome malfunctions and the hallmarks of pancreatic cancer. *Rev. Physiol. Biochem. Pharmacol.* [Epub ahead of print]. doi:10.1007/112_2020_20

Lioudyno, M. I., Kozak, J. A., Penna, A., Safrina, O., Zhang, S. L., Sen, D., et al. (2008). Orai1 and STIM1 move to the immunological synapse and are up-regulated during T-cell activation. *Proc. Natl. Acad. Sci. U.S.A.* 105, 2011–2016. doi:10.1073/pnas.0706122105

Liu, B. S., Ferreira, R., Lively, S., and Schlichter, L. C. (2013). Microglial SK3 and SK4 currents and activation state are modulated by the neuroprotective drug, riluzole. *J. Neuroimmune Pharmacol.* 8, 227–237. doi:10.1007/s11481-012-9365-0

Liu, J., Hu, G., Gong, Y., Yu, Q., He, B., Li, W., et al. (2018). Silencing of TRPM8 inhibits aggressive tumor phenotypes and enhances gemcitabine sensitivity in pancreatic cancer. *Pancreatol. Off. J. Int. Assoc. Pancreatol.* 18, 935–944. doi:10.1016/j.pan.2018.08.011

Liu, Q., Li, Y., Niu, Z., Zong, Y., Wang, M., Yao, L., et al. (2016). Atorvastatin (Lipitor) attenuates the effects of aspirin on pancreatic cancerogenesis and the chemotherapeutic efficacy of gemcitabine on pancreatic cancer by promoting M2 polarized tumor associated macrophages. *J. Exp. Clin. Cancer Res.* 35, 1–16. doi:10.1186/s13046-016-0304-4

Lohneis, P., Sinn, M., Bischoff, S., Jühling, A., Pelzer, U., Wislocka, L., et al. (2017). Cytotoxic tumour-infiltrating T lymphocytes influence outcome in resected pancreatic ductal adenocarcinoma. *Eur. J. Cancer.* 83, 290–301. doi:10.1016/j.ejca.2017.06.016

Luo, Y., Xue, Y., Wang, J., Dang, J., Fang, Q., Huang, G., et al. (2019). Negligible effect of sodium chloride on the development and function of TGF-β-induced CD4 + Foxp3 + regulatory T-cells. *Cell Rep.* 26, 1869–1879.e3 doi:10.1016/j.celrep.2019.01.066

Mace, T. A., Ameen, Z., Collins, A., Wojcik, S., Mair, M., Young, G. S., et al. (2013). Pancreatic cancer-associated stellate cells promote differentiation of myeloid-derived suppressor cells in a StAT3-dependent manner. *Cancer Res.* 73, 3007–3018. doi:10.1158/0008-5472.CAN-12-4601

Mahadevan, D., and Von Hoff, D. D. (2007). Tumor-stroma interactions in pancreatic ductal adenocarcinoma. *Mol. Cancer Ther.* 6, 1186–1197. doi:10.1158/1535-7163.MCT-06-0686

Maleki Vareki, S. (2018). High and low mutational burden tumors versus immunologically hot and cold tumors and response to immune checkpoint inhibitors. *J. Immunother. Canc.* 6, 157. doi:10.1186/s40425-018-0479-7

Manoli, S., Coppola, S., Duranti, C., Lulli, M., Magni, L., Kuppalu, N., et al. (2019). The activity of Kv 11.1 potassium channel modulates F-Actin organization during cell migration of pancreatic ductal adenocarcinoma cells. *Cancers*. 11, 135. doi:10.3390/cancers11020135

Marsh, J., Jackman, C. P., Tang, S.-N., Shankar, S., and Srivastava, R. K. (2014). Embelin suppresses pancreatic cancer growth by modulating tumor immune microenvironment. *Front. Biosci.* 19, 113. doi:10.2741/4198

Masamune, A., Kotani, H., Sörgel, F. L., Chen, J. M., Hamada, S., Sakaguchi, R., et al. (2020). Variants that affect function of calcium channel TRPV6 are associated with early-onset chronic pancreatitis. *Gastroenterology*. 158, 1626–1641.e8. doi:10.1053/j.gastro.2020.01.005

Masia, R., Krause, D. S., and Yellen, G. (2015). The inward rectifier potassium channel Kir2.1 is expressed in mouse neutrophils from bone marrow and liver. *Am. J. Physiol. – Cell Physiol.* 308, C264–C276. doi:10.1152/ajpcell.00176.2014

Matzner, N., Zemtsova, I. M., Xuan, N. T., Duszenko, M., Shumilina, E., and Lang, F. (2008). Ion channels modulating mouse dendritic cell functions. *J. Immunol.* 181, 6803–6809. doi:10.4049/jimmunol.181.10.6803

McAllister, F., Bailey, J. M., Alsina, J., Nirschl, C. J., Sharma, R., Fan, H., et al. (2014). Oncogenic kras activates a hematopoietic-to-epithelial IL-17 signaling axis in preinvasive pancreatic neoplasia. *Canc. Cell.* 25, 621–637. doi:10.1016/j.ccr.2014.03.014

McCarl, C.-A., Khalil, S., Ma, J., Oh-hora, M., Yamashita, M., Roether, J., et al. (2010). Store-operated Ca^{2+} entry through ORAI1 is critical for T cell-mediated autoimmunity and allograft rejection. *J. Immunol.* 185, 5845–5858. doi:10.4049/jimmunol.1001796

McDonald, B., Pittman, K., Menezes, G. B., Hirota, S. A., Slaba, I., Waterhouse, C. C. M., et al. (2010). Intravascular danger signals guide neutrophils to sites of sterile inflammation. *Science*. 330, 362–366. doi:10.1126/science.1195491

McKay, C. J., Glen, P., and McMillan, D. C. (2008). Chronic inflammation and pancreatic cancer. *Best Pract. Res. Clin. Gastroenterol.* 22, 65–73. doi:10.1016/j.bpg.2007.11.007

Meyer, R., and Heinemann, S. H. (1998). Characterization of an eag-like potassium channel in human neuroblastoma cells. *J. Physiol.* 508, 49–56. doi:10.1111/j.1469-7793.1998.049br.x

Meyer, R., Schönherr, R., Gavrilova-Ruch, O., Wohlrab, W., and Heinemann, S. H. (1999). Identification of ether a go-go and calcium-activated potassium channels in human melanoma cells. *J. Membr. Biol.* 171, 107–115. doi:10.1007/s002329900563

Mitcheson, J., and Arcangeli, A. (2014). "CHAPTER 11," in *The therapeutic potential of hERG1 K^+ channels for treating cancer and cardiac arrhythmias. (Royal Society of Chemistry)*, 258–296. doi:10.1039/9781849735087-00258

Miyai, Y., Esaki, N., Takahashi, M., and Enomoto, A. (2020). Cancer-associated fibroblasts that restrain cancer progression: hypotheses and perspectives. *Cancer Sci.* 111, 1047–1057. doi:10.1111/cas.14346

Mohammed, A., Janakiram, N. B., Madka, V., Pathuri, G., Li, Q., Ritchie, R., et al. (2017). Lack of chemopreventive effects of P2X7R inhibitors against pancreatic cancer. *Oncotarget*. 8, 97822–97834. doi:10.18632/oncotarget.22085

Mohr, C. J., Steudel, F. A., Gross, D., Ruth, P., Lo, W.-Y., Hoppe, R., et al. (2019). Cancer-associated intermediate conductance Ca^{2+}-activated K^+ channel $K_{Ca}3.1$. *Cancers*. 11, 109. doi:10.3390/cancers11010109

Møller, L. S., Fialla, A. D., Schierwagen, R., Biagini, M., Liedtke, C., Laleman, W., et al. (2016). The calcium-Activated potassium channel KCa3.1 is an important modulator of hepatic injury. *Sci. Rep.* 6, 1–12. doi:10.1038/srep28770

Morishita, K., Watanabe, K., and Ichijo, H. (2019). Cell volume regulation in cancer cell migration driven by osmotic water flow. *Canc. Sci.* 110, 2337–2347. doi:10.1111/cas.14079

Mroczko, B., Groblewska, M., Gryko, M., K□dra, B., and Szmitkowski, M. (2010). Diagnostic usefulness of serum Interleukin 6 (IL-6) and C-Reactive Protein (CRP) in the differentiation between pancreatic cancer and chronic pancreatitis. *J. Clin. Lab. Anal.* 24, 256–261. doi:10.1002/jcla.20395

Murugaiyan, G., and Saha, B. (2009). Protumor vs antitumor functions of IL-17. *J. Immunol.* 183, 4169–4175. doi:10.4049/jimmunol.0901017

Muthalagu, N., Monteverde, T., Raffo-Iraolagoitia, X., Wiesheu, R., Whyte, D., Hedley, A., et al. (2020). Repression of the type I interferon pathway underlies MYC & KRAS-dependent evasion of NK & B cells in pancreatic ductal adenocarcinoma. *Cancer Discov.* [Epub ahead of print] doi:10.1158/2159-8290.cd-19-0620

Mutini, C., Falzoni, S., Ferrari, D., Chiozzi, P., Morelli, A., Baricordi, O. R., et al. (1999). Mouse dendritic cells express the P2X7 purinergic receptor: characterization and possible participation in antigen presentation. *J. Immunol.* 163, 1958–1965.

Nagy, É., Mocsár, G., Sebestyén, V., Volkó, J., Papp, F., Tóth, K., et al. (2018). Membrane potential distinctly modulates mobility and signaling of IL-2 and IL-15 receptors in T-cells. *Biophys. J.* 114, 2473–2482. doi:10.1016/j.bpj.2018.04.038

Najder, K., Musset, B., Lindemann, O., Bulk, E., Schwab, A., and Fels, B. (2018). The function of TRP channels in neutrophil granulocytes. *Pflugers Arch.* 470, 1017–1033. doi:10.1007/s00424-018-2146-8

Nejati, R., Goldstein, J. B., Wang, H., Halperin, D. M., Wang, H., Hejazi, N., et al. (2017). Prognostic significance of tumor-infiltrating lymphocytes in patients with pancreatic ductal adenocarcinoma treated with neoadjuvant chemotherapy. *Pancreas*. 46, 1180–1187. doi:10.1097/MPA.0000000000000914

Nielsen, N., Kondratska, K., Ruck, T., Hild, B., Kovalenko, I., Schimmelpfennig, S., et al. (2017). TRPC6 channels modulate the response of pancreatic stellate cells to hypoxia. *Pflugers Arch. Eur. J. Physiol.* 469, 1567–1577. doi:10.1007/s00424-017-2057-0

Nilius, B., and Wohlrab, W. (1992). Potassium channels and regulation of proliferation of human melanoma cells. *J. Physiol.* 445, 537–548. doi:10.1113/jphysiol.1992.sp018938

Nywening, T. M., Belt, B. A., Cullinan, D. R., Panni, R. Z., Han, B. J., Sanford, D. E., et al. (2018). Targeting both tumour-associated CXCR2+ neutrophils and CCR2+ macrophages disrupts myeloid recruitment and improves chemotherapeutic responses in pancreatic ductal adenocarcinoma. *Gut.* 67, 1112–1123. doi:10.1136/gutjnl-2017-313738

Oberg, H. H., Wesch, D., Kalyan, S., and Kabelitz, D. (2019). Regulatory interactions between neutrophils, tumor cells and T-cells. *Front. Immunol.* 10, 1690. doi:10.3389/fimmu.2019.01690

Öhlund, D., Handly-Santana, A., Biffi, G., Elyada, E., Almeida, A. S., Ponz-Sarvise, M., et al. (2017). Distinct populations of inflammatory fibroblasts and myofibroblasts in pancreatic cancer. *J. Exp. Med.* 214, 579–596. doi:10.1084/jem.20162024

Ohya, S., and Kito, H. (2018). Ca2+-Activated K+ channel KCa3.1 as a therapeutic target for immune disorders. *Biol. Pharm. Bull.* 41, 1158–1163. doi:10.1248/bpb.b18-00078

Olive, K. P., Jacobetz, M. a., Davidson, C. J., Gopinathan, A., McIntyre, D., Honess, D., et al. (2011). Inhibition of Hedgehog signaling. *Science*. 324, 1457–1461. doi:10.1126/science.1171362

Olive, K. P., Jacobetz, M. A., Davidson, C. J., Gopinathan, A., McIntyre, D., Honess, D., et al. (2009). Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science*. 324, 1457–1461. doi:10.1126/science.1171362

Omary, M. B., Lowe, A. W., Pandol, S. J., Omary, M. B., Lugea, A., Lowe, A. W., et al. (2007a). The pancreatic stellate cell : a star on the rise in pancreatic diseases find the latest version : review series the pancreatic stellate cell : a star on the rise in pancreatic diseases. *J. Clin. Invest.* 117, 50–59. doi:10.1172/JCI30082.50

Omary, M. B., Lugea, A., Lowe, A. W., and Pandol, S. J. (2007b). The pancreatic stellate cell: a star on the rise in pancreatic diseases. *J. Clin. Invest.* 117, 50–59. doi:10.1172/JCI30082

Ong, S. T., Ng, A. S., Ng, X. R., Zhuang, Z., Wong, B. H. S., Prasannan, P., et al. (2019). Extracellular K^+ dampens T-cell functions: implications for immune suppression in the tumor microenvironment. *Bioelectricity*. 1, 169–179. doi:10.1089/bioe.2019.0016

Orbán, C., Bajnok, A., Vásárhelyi, B., Tulassay, T., and Toldi, G. (2014). Different calcium influx characteristics upon Kv1.3 and IKCa1 potassium channel inhibition in T helper subsets. *Cytom. Part A*. 85, 636–641. doi:10.1002/cyto.a.22479

Ouadid-Ahidouch, H., Le Bourhis, X., Roudbaraki, M., Toillon, R. A., Delcourt, P., and Prevarskaya, N. (2001). Changes in the K+ current-density of MCF-7 cells during progression through the cell cycle: possible Involvement of a h-ether.a-gogo K+ channel. *Recept. Chann.* 7, 345–356.

Ousingsawat, J., Spitzner, M., Puntheeranurak, S., Terracciano, L., Tornillo, L., Bubendorf, L., et al. (2007). Expression of voltage-gated potassium channels in

human and mouse colonic carcinoma. *Clin. Cancer Res.* 13, 824–831. doi:10.1158/1078-0432.CCR-06-1940

Özdemir, B. C., Pentcheva-Hoang, T., Carstens, J. L., Zheng, X., Wu, C. C., Simpson, T. R., et al. (2014). Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival. *Canc. Cell.* 25, 719–734. doi:10.1016/j.ccr.2014.04.005

Pahl, A., Lutz, C., and Hechler, T. (2018). Amanitins and their development as a payload for antibody-drug conjugates. *Drug Discov. Today. Technol.* 30, 85–89. doi:10.1016/j.ddtec.2018.08.005

Panyi, G., Beeton, C., and Felipe, A. (2014). Ion channels and anti-cancer immunity. *Philos. Trans. R. Soc. B Biol. Sci.* 369, 20130106. doi:10.1098/rstb.2013.0106

Panyi, G., Possani, L., de la Vega, R. C., Gaspar, R., and Varga, Z. (2006). K+ channel blockers: novel tools to inhibit T-cell activation leading to specific immunosuppression. *Curr. Pharm. Des.* 12, 2199–2220. doi:10.2174/138161206777585120

Pardo, L. A., and Stühmer, W. (2014). The roles of K^+ channels in cancer. *Nat. Rev. Canc.* 14, 39–48. doi:10.1038/nrc3635

Pardo, L. A., Del Camino, D., Sánchez, A., Alves, F., Brüggemann, A., Beckh, S., et al. (1999). Oncogenic potential of EAG K^+ channels. *EMBO J.* 18, 5540–5547. doi:10.1093/emboj/18.20.5540

Parslow, A. C., Parakh, S., Lee, F.-T., Gan, H. K., and Scott, A. M. (2016). Antibody-drug conjugates for cancer therapy. *Biomedicines.* 4, 14. doi:10.3390/biomedicines4030014

Pedersen, S. F., Novak, I., Alves, F., Schwab, A., and Pardo, L. A. (2017). Alternating pH landscapes shape epithelial cancer initiation and progression: focus on pancreatic cancer. *BioEssays.* 39. doi:10.1002/bies.201600253

Peixoto, R. D. A., Speers, C., Mcgahan, C. E., Renouf, D. J., Schaeffer, D. F., and Kennecke, H. F. (2015). Prognostic factors and sites of metastasis in unresectable locally advanced pancreatic cancer. *Canc. Med.* 4, 1171–1177. doi:10.1002/cam4.459

Pethő, Z., Najder, K., Bulk, E., and Schwab, A. (2019). Mechanosensitive ion channels push cancer progression. *Cell Calcium.* 80, 79–90. doi:10.1016/j.ceca.2019.03.007

Pethő, Z., Najder, K., Carvalho, T., McMorrow, R., Todesca, L. M., Rugi, M., et al. (2020). pH-channeling in cancer: how pH-dependence of cation channels shapes cancer pathophysiology. *Cancers.* 12, 2484. doi:10.3390/cancers12092484

Petroni, G., Bagni, G., Iorio, J., Duranti, C., Lottini, T., Stefanini, M., et al. (2020). Clarithromycin inhibits autophagy in colorectal cancer by regulating the hERG1 potassium channel interaction with PI3K. *Cell Death Dis.* 11, 161. doi:10.1038/s41419-020-2349-8

Pillozzi, S., Brizzi, M. F., Balzi, M., Crociani, O., Cherubini, A., Guasti, L., et al. (2002). HERG potassium channels are constitutively expressed in primary human acute myeloid leukemias and regulate cell proliferation of normal and leukemic hemopoietic progenitors. *Leukemia.* 16, 1791–1798. doi:10.1038/sj.leu.2402572

Pillozzi, S., D'Amico, M., Bartoli, G., Gasparoli, L., Petroni, G., Crociani, O., et al. (2018). The combined activation of $K_{Ca}3.1$ and inhibition of $K_V11.1$/hERG1 currents contribute to overcome Cisplatin resistance in colorectal cancer cells. *Br. J. Cancer.* 118, 200–212. doi:10.1038/bjc.2017.392

Piro, G., Simionato, F., Carbone, C., Frizziero, M., Malleo, G., Zanini, S., et al. (2017). A circulating TH2 cytokines profile predicts survival in patients with resectable pancreatic adenocarcinoma. *Oncoimmunology.* 6, e1322242. doi:10.1080/2162402X.2017.1322242

Pointer, K. B., Clark, P. A., Eliceiri, K. W., Salamat, M. S., Robertson, G. A., and Kuo, J. S. (2017). Administration of non-torsadogenic human Ether-à-go-go-Related Gene inhibitors is associated with better survival for high hERG-expressing glioblastoma patients. *Clin. Cancer Res.* 23, 73–80. doi:10.1158/1078-0432.CCR-15-3169

Poschke, I., Faryna, M., Bergmann, F., Flossdorf, M., Lauenstein, C., Hermes, J., et al. (2016). Identification of a tumor-reactive T-cell repertoire in the immune in fi ltrate of patients with resectable pancreatic ductal adenocarcinoma. *Oncoimmunology.* 5, e1240859. doi:10.1080/2162402X.2016.1240859

Prevarskaya, N., Skryma, R., and Shuba, Y. (2018). Ion channels in cancer: are cancer hallmarks oncochannelopathies?. *Physiol. Rev.* 98, 559–621. doi:10.1152/physrev.00044.2016

Provenzano, P. P., Cuevas, C., Chang, A. E., Goel, V. K., Von Hoff, D. D., and Hingorani, S. R. (2012). Enzymatic targeting of the stroma ablates physical barriers to treatment of pancreatic ductal adenocarcinoma. *Canc. Cell.* 21, 418–429. doi:10.1016/j.ccr.2012.01.007

Puche, J. E., Saiman, Y., and Friedman, S. L. (2013). Hepatic stellate cells and liver fibrosis. *Compr. Physiol.* 3, 1473–1492. doi:10.1002/cphy.c120035

Rahaman, S. O., Grove, L. M., Paruchuri, S., Southern, B. D., Abraham, S., Niese, K. A., et al. (2014). TRPV4 mediates myofibroblast differentiation and pulmonary fibrosis in mice. *J. Clin. Invest.* 124, 5225–5238. doi:10.1172/JCI75331

Ramsey, I. S., Ruchti, E., Kaczmarek, J. S., and Clapham, D. E. (2009). Hv1 proton channels are required for high-level NADPH oxidase-dependent superoxide production during the phagocyte respiratory burst. *Proc. Natl. Acad. Sci. U.S.A.* 106, 7642–7647. doi:10.1073/pnas.0902761106

Raphaël, M., Lehen'kyi, V., Vandenberghe, M., Beck, B., Khalimonchyk, S., Vanden Abeele, F., et al. (2014). TRPV6 calcium channel translocates to the plasma membrane via Orai1-mediated mechanism and controls cancer cell survival. *Proc. Natl. Acad. Sci. U.S.A.* 111, E3870–E3879. doi:10.1073/pnas.1413409111

Rawla, P., Sunkara, T., and Gaduputi, V. (2019). Epidemiology of pancreatic cancer: global trends, Etiology and risk factors. *World J. Oncol.* 10, 10–27. doi:10.14740/wjon1166

Redmond, J., and Buchanan, P. (2017). Role of ion channels in natural killer cell function towards cancer. *Discov. Med.* 23, 353–360.

Reneer, M. C., Estes, D. J., Vélez-Ortega, A. C., Norris, A., Mayer, M., and Marti, F. (2011). Peripherally induced human regulatory T-cells uncouple Kv1.3 activation from TCR-associated signaling. *Eur. J. Immunol.* 41, 3170–3175. doi:10.1002/eji.201141492

Roach, K. M., and Bradding, P. (2019). Ca^{2+} signalling in fibroblasts and the therapeutic potential of $K_{Ca}3.1$ channel blockers in fibrotic diseases. *Br. J. Pharmacol.* 177, 1003–1024. doi:10.1111/bph.14939

Roach, K. M., Duffy, S. M., Coward, W., Feghali-Bostwick, C., Wulff, H., and Bradding, P. (2013). The K^+ channel $K_{Ca}3.1$ as a novel target for idiopathic pulmonary fibrosis. *PLoS One.* 8, e85244. doi:10.1371/journal.pone.0085244

Rodrigues, A. R. A., Arantes, E. C., Monje, F., Stühmer, W., and Varanda, W. A. (2003). Tityustoxin-K(alpha) blockade of the voltage-gated potassium channel Kv1.3. *Br. J. Pharmacol.* 139, 1180–1186. doi:10.1038/sj.bjp.0705343

Roghanian, A., Fraser, C., Kleyman, M., and Chen, J. (2016). B cells promote pancreatic tumorigenesis. *Canc. Discov.* 6, 230–232. doi:10.1158/2159-8290.CD-16-0100

Rybarczyk, P., Gautier, M., Hague, F., Dhennin-Duthille, I., Chatelain, D., Kerr-Conte, J., et al. (2012). Transient receptor potential melastatin-related 7 channel is overexpressed in human pancreatic ductal adenocarcinomas and regulates human pancreatic cancer cell migration. *Int. J. Cancer.* 131, 851–861. doi:10.1002/ijc.27487

Rybarczyk, P., Vanlaeys, A., Brassart, B., Dhennin-Duthille, I., Chatelain, D., Sevestre, H., et al. (2017). The transient receptor potential melastatin 7 channel regulates pancreatic cancer cell invasion through the hsp90α/uPA/MMP2 pathway. *Neoplasia (United States).* 19, 288–300. doi:10.1016/j.neo.2017.01.004

Saéz, P. J., Vargas, P., Shoji, K. F., Harcha, P. A., Lennon-Duméni, A. M., and Saéz, J. C. (2017). ATP promotes the fast migration of dendritic cells through the activity of pannexin 1 channels and P2X7 receptors. *Sci. Signal.* 10, eaah7107. doi:10.1126/scisignal.aah7107

Sakata, R., Ueno, T., Nakamura, T., Ueno, H., and Sata, M. (2004). Mechanical stretch induces TGF-β synthesis in hepatic stellate cells. *Eur. J. Clin. Invest.* 34, 129–136. doi:10.1111/j.1365-2362.2004.01302.x

Sakellariou-Thompson, D., Forget, M. A., Creasy, C., Bernard, V., Zhao, L., Kim, Y. U., et al. (2017). 4-1BB agonist focuses CD8+ tumor-infiltrating T-Cell growth into a distinct repertoire capable of tumor recognition in pancreatic cancer. *Clin. Cancer Res.* 23, 7263–7275. doi:10.1158/1078-0432.CCR-17-0831

Sanguinetti, M. C. (2010). HERG1 channelopathies. *Pflugers Arch. Eur. J. Physiol.* 460, 265–276. doi:10.1007/s00424-009-0758-8

Sanguinetti, M. C., Jiang, C., Curran, M. E., and Keating, M. T. (1995). A mechanistic link between an inherited and an acquired cardiac arrhythmia: HERG encodes the IKr potassium channel. *Cell.* 81, 299–307. doi:10.1016/0092-8674(95)90340-2

Saurer, L., Reber, P., Schaffner, T., Büchler, M. W., Buri, C., Kappeler, A., et al. (2000). Differential expression of chemokines in normal pancreas and in chronic pancreatitis. *Gastroenterology.* 118, 356–367. doi:10.1016/S0016-5085(00)70218-6

Sauter, D. R. P., Novak, I., Pedersen, S. F., Larsen, E. H., and Hoffmann, E. K. (2015). ANO1 (TMEM16A) in pancreatic ductal adenocarcinoma (PDAC). *Pflugers Arch. Eur. J. Physiol.* 467, 1495–1508. doi:10.1007/s00424-014-1598-8

Sauter, D. R. P., Sørensen, C. E., Rapedius, M., Brüggemann, A., and Novak, I. (2016). pH-sensitive K+ channel TREK-1 is a novel target in pancreatic cancer. *Biochim. Biophys. Acta – Mol. Basis Dis.* 1862, 1994–2003. doi:10.1016/j.bbadis.2016.07.009

Schappe, M. S., Szteyn, K., Stremska, M. E., Mendu, S. K., Downs, T. K., Seegren, P. V., et al. (2018). Chanzyme TRPM7 mediates the Ca2+ influx essential for lipopolysaccharide-induced toll-like receptor 4 Endocytosis and macrophage activation. *Immunity*. 48, 59–74. doi:10.1016/j.immuni.2017.11.026.e5

Schilling, T., Miralles, F., and Eder, C. (2014). TRPM7 regulates proliferation and polarisation of macrophages. *J. Cell Sci.* 127, 4561–4566. doi:10.1242/jcs.151068

Schnipper, J., Dhennin-duthille, I., and Ahidouch, A. (2020). Ion channel signature in healthy pancreas and pancreatic ductal adenocarcinoma. *Front. Pharmacol.* 11, 568993. doi:10.3389/fphar.2020.568993

Schwab, A., and Stock, C. (2014). Ion channels and transporters in tumour cell migration and invasion. *Philos. Trans. R. Soc. B Biol. Sci.* 369, 20130102. doi:10.1098/rstb.2013.0102

Schwab, A., Nechyporuk-Zloy, V., Gassner, B., Schulz, C., Kessler, W., Mally, S., et al. (2012). Dynamic redistribution of calcium sensitive potassium channels $hK_{Ca}3.1$ in migrating cells. *J. Cell. Physiol.* 227, 686–696. doi:10.1002/jcp.22776

Schwab, A., Wulf, A., Schulz, C., Kessler, W., Nechyporuk-Zloy, V., Römer, M., et al. (2006). Subcellular distribution of calcium-sensitive potassium channels (Ik1) in migrating cells. *J. Cell. Physiol.* 206, 86–94. doi:10.1002/jcp.20434

Segerstolpe, Å., Palasantza, A., Eliasson, P., Andersson, E.-M., Andréasson, A.-C., Sun, X., et al. (2016). Single-cell transcriptome profiling of human pancreatic islets in health and type 2 diabetes. *Cell Metab.* 24, 593–607. doi:10.1016/j.cmet.2016.08.020

Shao, P. P., Liu, C. J., Xu, Q., Zhang, B., Li, S. H., Wu, Y., et al. (2018). Eplerenone reverses cardiac fibrosis via the suppression of Tregs by inhibition of Kv1.3 channel. *Front. Physiol.* 9, 899. doi:10.3389/fphys.2018.00899

Sharma, S., Goswami, R., Zhang, D. X., and Rahaman, S. O. (2019). TRPV4 regulates matrix stiffness and TGFβ1-induced epithelial-mesenchymal transition. *J. Cell. Mol. Med.* 23, 761–774. doi:10.1111/jcmm.13972

Shevchenko, I., Karakhanova, S., Soltek, S., Link, J., Bayry, J., Werner, J., et al. (2013). Low-dose gemcitabine depletes regulatory T-cells and improves survival in the orthotopic Panc02 model of pancreatic cancer. *Int. J. Cancer.* 133, 98–107. doi:10.1002/ijc.27990

Shi, Y., Sahu, R. P., and Srivastava, S. K. (2008). Triphala inhibits both *in vitro* and *in vivo* xenograft growth of pancreatic tumor cells by inducing apoptosis. *BMC Canc.* 8, 294. doi:10.1186/1471-2407-8-294

Shurin, M. R., Lu, L., Kalinski, P., Stewart-Akers, A. M., and Lotze, M. T. (1999). Th1/Th2 balance in cancer, transplantation and pregnancy. *Springer Semin Immunopathol.* 21, 339–359. doi:10.1007/BF00812261

Silva-Santos, J. E. D. A., Santos-Silva, M. C., De Queiroz Cunha, F., and Assreuy, J. (2002). The role of ATP-sensitive potassium channels in neutrophil migration and plasma exudation. *J. Pharmacol. Exp. Therapeut.* 300, 946–951. doi:10.1124/jpet.300.3.946

Singh, R. R., and O'Reilly, E. M. (2020). New treatment strategies for metastatic pancreatic ductal adenocarcinoma. *Drugs.* 80, 647–669. doi:10.1007/s40265-020-01304-0

Somodi, S., Hajdu, P., Gáspár, R., Panyi, G., and Varga, Z. (2008). Effects of changes in extracellular pH and potassium concentration on Kv1.3 inactivation. *Eur. Biophys. J.* 37, 1145–1156. doi:10.1007/s00249-008-0267-2

Somodi, S., Varga, Z., Hajdu, P., Starkus, J. G., Levy, D. I., Gáspár, R., et al. (2004). pH-dependent modulation of Kv1.3 inactivation: role of His399. *Am. J. Physiol. – Cell Physiol.* 287, C1067–C1076. doi:10.1152/ajpcell.00438.2003

Song, H., Dong, M., Zhou, J., Sheng, W., Li, X., and Gao, W. (2018). Expression and prognostic significance of TRPV6 in the development and progression of pancreatic cancer. *Oncol. Rep.* 39, 1432–1440. doi:10.3892/or.2018.6216

Song, J. H., Huang, C. S., Nagata, K., Yeh, J. Z., and Narahashi, T. (1997). Differential action of riluzole on tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels. *J. Pharmacol. Exp. Therapeut.* 282, 707–714.

Songa, Y., Zhan, L., Yu, M., Huang, C., Meng, X., Taotao, M., et al. (2014). TRPV4 channel inhibits TGF-β1-induced proliferation of hepatic stellate cells. *PLoS One.* 9, e101179. doi:10.1371/journal.pone.0101179

Spear, S., Candido, J. B., McDermott, J. R., Ghirelli, C., Maniati, E., Beers, S. A., et al. (2019). Discrepancies in the tumor microenvironment of spontaneous and orthotopic murine models of pancreatic cancer uncover a new immunostimulatory phenotype for B cells. *Front. Immunol.* 10, 542. doi:10.3389/fimmu.2019.00542

Sperb, N., Tsesmelis, M., and Wirth, T. (2020). Crosstalk between tumor and stromal cells in pancreatic ductal adenocarcinoma. *Int. J. Mol. Sci.* 21, 5486. doi:10.3390/ijms21155486

Starkus, J. G., Varga, Z., Schönherr, R., and Heinemann, S. H. (2003). Mechanisms of the inhibition of Shaker potassium channels by protons. *Pflugers Arch. Eur. J. Physiol.* 447, 44–54. doi:10.1007/s00424-003-1121-0

Steele, C. W., Karim, S. A., Leach, J. D. G., Bailey, P., Upstill-Goddard, R., Rishi, L., et al. (2016). CXCR2 inhibition profoundly suppresses metastases and augments immunotherapy in pancreatic ductal adenocarcinoma. *Canc. Cell.* 29, 832–845. doi:10.1016/j.ccell.2016.04.014

Steudel, F. A., Mohr, C. J., Stegen, B., Nguyen, H. Y., Barnert, A., Steinle, M., et al. (2017). SK4 channels modulate Ca(2+) signalling and cell cycle progression in murine breast cancer. *Mol. Oncol.* 11, 1172–1188. doi:10.1002/1878-0261.12087

Storck, H., Hild, B., Schimmelpfennig, S., Sargin, S., Nielsen, N., Zaccagnino, A., et al. (2017). Ion channels in control of pancreatic stellate cell migration. *Oncotarget.* 8, 769–784. doi:10.18632/oncotarget.13647

Stromnes, I. M., Hulbert, A., Pierce, R. H., Greenberg, P. D., and Hingorani, S. R. (2017). T-cell localization, activation, and clonal expansion in human pancreatic ductal adenocarcinoma. *Cancer Immunol. Res.* 5, 978–991. doi:10.1158/2326-6066.CIR-16-0322

Stylianopoulos, T., Martin, J. D., Chauhan, V. P., Jain, S. R., Diop-Frimpong, B., Bardeesy, N., et al. (2012). Causes, consequences, and remedies for growth-induced solid stress in murine and human tumors. *Proc. Natl. Acad. Sci. U.S.A.* 109, 15101–15108. doi:10.1073/pnas.1213353109

Sun, Q., Zhou, C., Ma, R., Guo, Q., Huang, H., Hao, J., et al. (2018). Prognostic value of increased integrin-beta 1 expression in solid cancers: a meta-analysis. *Onco Targets Ther.* 11, 1787–1799. doi:10.2147/OTT.S155279

Sun, R., He, X., Jiang, X., and Tao, H. (2019). The new role of riluzole in the treatment of pancreatic cancer through the apoptosis and autophagy pathways. *J. Cell. Biochem.* [Epub ahead of print]. doi:10.1002/jcb.29533

Sunami, Y., and Kleeff, J. (2019). Immunotherapy of pancreatic cancer. *Prog. Mol. Biol. Transl. Sci.* 164, 189–216. doi:10.1016/bs.pmbts.2019.03.006

Szteyn, K., Yang, W., Schmid, E., Lang, F., and Shumilina, E. (2012). Lipopolysaccharide-sensitive H^+ current in dendritic cells. *Am. J. Physiol. - Cell Physiol.* 303, 204–212. doi:10.1152/ajpcell.00059.2012

Tajti, G., Wai, D. C. C., Panyi, G., and Norton, R. S. (2020). The voltage-gated potassium channel $K_V1.3$ as a therapeutic target for venom-derived peptides. *Biochem. Pharmacol.* 114146. doi:10.1016/j.bcp.2020.114146

Takesue, S., Ohuchida, K., Shinkawa, T., Otsubo, Y., Matsumoto, S., Sagara, A., et al. (2020). Neutrophil extracellular traps promote liver micrometastasis in pancreatic ductal adenocarcinoma via the activation of cancer-associated fibroblasts. *Int. J. Oncol.* 56, 596–605. doi:10.3892/ijo.2019.4951

Tang, Y., Xu, X., Guo, S., Zhang, C., Tang, Y., Tian, Y., et al. (2014). An increased abundance of tumor-infiltrating regulatory t cells is correlated with the progression and prognosis of pancreatic ductal adenocarcinoma. *PLoS One.* 9, e91551. doi:10.1371/journal.pone.0091551

Tao, L., Zhang, L., Peng, Y., Tao, M., Li, L., Xiu, D., et al. (2016). Neutrophils assist the metastasis of circulating tumor cells in pancreatic ductal adenocarcinoma: a new hypothesis and a new predictor for distant metastasis. *Medicine (Baltimore).* 95, e4932. doi:10.1097/MD.0000000000004932

Tawfik, D., Zaccagnino, A., Bernt, A., Szczepanowski, M., Klapper, W., Schwab, A., et al. (2020). The A818-6 system as an *in-vitro* model for studying the role of the transportome in pancreatic cancer. *BMC Canc.* 20, 264. doi:10.1186/s12885-020-06773-w

Thyagarajan, A., Alshehri, M. S. A., Miller, K. L. R., Sherwin, C. M., Travers, J. B., and Sahu, R. P. (2019). Myeloid-derived suppressor cells and pancreatic cancer: implications in novel therapeutic approaches. *Cancers.* 11, 1627. doi:10.3390/cancers11111627

Toldi, G., Folyovich, A., Simon, Z., Zsiga, K., Kaposi, A., Mészáros, G., et al. (2011). Lymphocyte calcium influx kinetics in multiple sclerosis treated without or with interferon beta. *J. Neuroimmunol.* 237, 80–86. doi:10.1016/j.jneuroim.2011.06.008

Toldi, G., Kaposi, A., Zsembery, Á., Treszl, A., Tulassay, T., and Vásárhelyi, B. (2012). Human Th1 and Th2 lymphocytes are distinguished by calcium flux regulation during the first 10min of lymphocyte activation. *Immunobiology*. 217, 37–43. doi:10.1016/j.imbio.2011.08.007

Trovato, R., Fiore, A., Sartori, S., Canè, S., Giugno, R., Cascione, L., et al. (2019). Immunosuppression by monocytic myeloid-derived suppressor cells in patients with pancreatic ductal carcinoma is orchestrated by STAT3. *J. Immunother. Canc.* 7, 255. doi:10.1186/s40425-019-0734-6

Trudeau, M. C., Warmke, J. W., Ganetzky, B., and Robertson, G. A. (1995). HERG, a human inward rectifier in the voltage-gated potassium channel family. *Science*. 269, 92–95. doi:10.1126/science.7604285

Urrego, D., Tomczak, A. P., Zahed, F., Stühmer, W., and Pardo, L. A. (2014). Potassium channels in cell cycle and cell proliferation. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 369, 20130094. doi:10.1098/rstb.2013.0094

Vaeth, M., Wang, Y. H., Eckstein, M., Yang, J., Silverman, G. J., Lacruz, R. S., et al. (2019). Tissue resident and follicular Treg cell differentiation is regulated by CRAC channels. *Nat. Commun.* 10, 1183. doi:10.1038/s41467-019-08959-8

Vaeth, M., Yang, J., Yamashita, M., Zee, I., Eckstein, M., Knosp, C., et al. (2017). ORAI2 modulates store-operated calcium entry and T cell-mediated immunity. *Nat. Commun.* 8, 14714. doi:10.1038/ncomms14714

Varga, Z., Csepany, T., Papp, F., Fabian, A., Gogolak, P., Toth, A., et al. (2009). Potassium channel expression in human CD4+ regulatory and naïve T-cells from healthy subjects and multiple sclerosis patients. *Immunol. Lett.* 124, 95–101. doi:10.1016/j.imlet.2009.04.008

Vesely, M. D., Kershaw, M. H., Schreiber, R. D., and Smyth, M. J. (2011). Natural innate and adaptive immunity to cancer. *Annu. Rev. Immunol.* 29, 235–271. doi:10.1146/annurev-immunol-031210-101324

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

Von Ahrens, D., Bhagat, T. D., Nagrath, D., Maitra, A., and Verma, A. (2017). The role of stromal cancer-associated fibroblasts in pancreatic cancer. *J. Hematol. Oncol.* 10, 76. doi:10.1186/s13045-017-0448-5

Wang, J., Haanes, K. A., and Novak, I. (2013). Purinergic regulation of CFTR and Ca2+-activated Cl-channels and K^+ channels in human pancreatic duct epithelium. *Am. J. Physiol. – Cell Physiol.* 304, 673–684. doi:10.1152/ajpcell.00196.2012

Wang, Q., Bai, L., Luo, S., Wang, T., Yang, F., Xia, J., et al. (2020a). TMEM16A Ca2+-activated Cl− channel inhibition ameliorates acute pancreatitis via the IP3R/Ca2+/NFκB/IL-6 signaling pathway. *J. Adv. Res.* 23, 25–35. doi:10.1016/j.jare.2020.01.006

Wang, S., Li, Y., Xing, C., Ding, C., Zhang, H., Chen, L., et al. (2020b). Tumor microenvironment in chemoresistance, metastasis and immunotherapy of pancreatic cancer. *Am. J. Cancer Res.* 10, 1937–1953.

Wang, X., Lang, M., Zhao, T., Feng, X., Zheng, C., Huang, C., et al. (2017). Cancer-FOXP3 directly activated CCL5 to recruit $FOXP3^+$ Treg cells in pancreatic ductal adenocarcinoma. *Oncogene*. 36, 3048–3058. doi:10.1038/onc.2016.458

Wang, Y., Fang, T., Huang, L., Wang, H., Zhang, L., Wang, Z., et al. (2018). Neutrophils infiltrating pancreatic ductal adenocarcinoma indicate higher malignancy and worse prognosis. *Biochem. Biophys. Res. Commun.* 501, 313–319. doi:10.1016/j.bbrc.2018.05.024

Weber, K. S., Hildner, K., Murphy, K. M., and Allen, P. M. (2010). Trpm4 differentially regulates Th1 and Th2 function by altering calcium signaling and NFAT localization. *J. Immunol.* 185, 2836–2846. doi:10.4049/jimmunol.1000880

Weisberg, S. P., Carpenter, D. J., Chait, M., Dogra, P., Gartrell-Corrado, R. D., Chen, A. X., et al. (2019). Tissue-Resident memory T-cells mediate immune homeostasis in the human pancreas through the PD-1/PD-L1 pathway. *Cell Rep.* 29, 3916–3932. doi:10.1016/j.celrep.2019.11.056.e5

Wen, Z., Liao, Q., Zhao, J., Hu, Y., You, L., Lu, Z., et al. (2014). High expression of interleukin-22 and its receptor predicts poor prognosis in pancreatic ductal adenocarcinoma. *Ann. Surg. Oncol.* 21, 125–132. doi:10.1245/s10434-013-3322-x

Whiteside, T. (2015). The role of regulatory T-cells in cancer immunology. *ImmunoTargets Ther.* 4, 159–171. doi:10.2147/itt.s55415

Willebrand, R., Hamad, I., Van Zeebroeck, L., Kiss, M., Bruderek, K., Geuzens, A., et al. (2019). High salt inhibits tumor growth by enhancing anti-tumor immunity. *Front. Immunol.* 10, 1–15. doi:10.3389/fimmu.2019.01141

Williams, S., Bateman, A., and O'Kelly, I. (2013). Altered expression of two-pore domain potassium (K2P) channels in cancer. *PLoS One*. 8, e74589. doi:10.1371/journal.pone.0074589

Wörmann, S. M., Diakopoulos, K. N., Lesina, M., and Algül, H. (2014). The immune network in pancreatic cancer development and progression. *Oncogene*. 33, 2956–2967. doi:10.1038/onc.2013.257

Wu, L., Saxena, S., Awaji, M., and Singh, R. K. (2019). Tumor-associated neutrophils in Cancer : going pro. *Cancers*. 11, 564. doi:10.3390/cancers11040564

Wulff, H., Calabresi, P. A., Allie, R., Yun, S., Pennington, M., Beeton, C., et al. (2003). The voltage-gated Kv1.3 K+ channel in effector memory T-cells as new target for MS. *J. Clin. Invest.* 111, 1703–1713. doi:10.1172/JCI16921

Wulff, H., Castle, N. A., and Pardo, L. A. (2009). Voltage-gated potassium channels as therapeutic targets. *Nat. Rev. Drug Discov.* 8, 982–1001. doi:10.1038/nrd2983

Wulff, H., Knaus, H.-G., Pennington, M., and Chandy, K. G. (2004). K + channel expression during B cell differentiation: implications for immunomodulation and autoimmunity. *J. Immunol.* 173, 776–786. doi:10.4049/jimmunol.173.2.776

Wulff, H., Kolski-Andreaco, A., Sankaranarayanan, A., Sabatier, J.-M., and Shakkottai, V. (2007). Modulators of small- and intermediate-conductance calcium-activated potassium channels and their therapeutic indications. *Curr. Med. Chem.* 14, 1437–1457. doi:10.2174/092986707780831186

Wulff, H., Miller, M. J., Hänsel, W., Grissmer, S., Cahalan, M. D., and Chandy, K. G. (2000). Design of a potent and selective inhibitor of the intermediate-conductance Ca^{2+}-activated K^+ channel, IKCa1: a potential immunosuppressant. *Proc. Natl. Acad. Sci. U.S.A.* 97, 8151–8156. doi:10.1073/pnas.97.14.8151

Wynn, T. A., and Ramalingam, T. R. (2012). Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat. Med.* 18, 1028–1040. doi:10.1038/nm.2807

Xu, R., Li, C., Wu, Y., Shen, L., Ma, J., Qian, J., et al. (2017). Role of $K_{Ca}3.1$ channels in macrophage polarization and its relevance in atherosclerotic plaque instability. *Arterioscler. Thromb. Vasc. Biol.* 37, 226–236. doi:10.1161/ATVBAHA.116.308461

Xu, T., Du, X. W., Hu, J. B., Zhu, Y. F., Wu, H. L., Dai, G. P., et al. (2018). Anticancer effect of miR-96 inhibitor in bladder cancer cell lines. *Oncol. Lett.* 15, 3814–3819. doi:10.3892/ol.2018.7745

Yako, Y. Y., Kruger, D., Smith, M., and Brand, M. (2016). Cytokines as biomarkers of pancreatic ductal adenocarcinoma: a systematic review. *PLoS One*. 11, e0154016. doi:10.1371/journal.pone.0154016

Yamamoto, S., Shimizu, S., Kiyonaka, S., Takahashi, N., Wajima, T., Hara, Y., et al. (2008). TRPM2-mediated Ca2+ influx induces chemokine production in monocytes that aggravates inflammatory neutrophil infiltration. *Nat. Med.* 14, 738–747. doi:10.1038/nm1758

Yang, D., Zhang, Q., Ma, Y., Che, Z., Zhang, W., Wu, M., et al. (2019). Augmenting the therapeutic efficacy of adenosine against pancreatic cancer by switching the Akt/p21-dependent senescence to apoptosis. *EBioMedicine*. 47, 114–127. doi:10.1016/j.ebiom.2019.08.068

Yee, N. S. (2017). Role of TRPM7 in cancer: potential as molecular biomarker and therapeutic target. *Pharmaceuticals*. 10, 39. doi:10.3390/ph10020039

Yee, N. S, Zhou, W., and Lee, M. (2010). Transient receptor potential channel TRPM8 is over-expressed and required for cellular proliferation in pancreatic adenocarcinoma. *Cancer Lett.* 297, 149–155. doi:10.1038/jid.2014.371

Yee, N. S., Kazi, A. A., Li, Q., Yang, Z., Berg, A., and Yee, R. K. (2015). Aberrant over-expression of TRPM7 ion channels in pancreatic cancer: required for cancer cell invasion and implicated in tumor growth and metastasis. *Biol. Open*. 4, 507–514. doi:10.1242/bio.20137088

Yee, N. S., Zhou, W., and Liang, I.-C. (2011). Transient receptor potential ion channel Trpm7 regulates exocrine pancreatic epithelial proliferation by Mg2+-sensitive Socs3a signaling in development and cancer. *Dis. Model. Mech.* 4, 240–254. doi:10.1242/dmm.004564

Zaccagnino, A., Managò, A., Leanza, L., Gontarewitz, A., Linder, B., Azzolini, M., et al. (2017). Tumor-reducing effect of the clinically used drug clofazimine in a SCID mouse model of pancreatic ductal adenocarcinoma. *Oncotarget*. 8, 38276–38293. doi:10.18632/oncotarget.11299

Zaccagnino, A., Pilarsky, C., Tawfik, D., Sebens, S., Trauzold, A., Novak, I., et al. (2016). In silico analysis of the transportome in human pancreatic ductal adenocarcinoma. *Eur. Biophys. J.* 45, 749–763. doi:10.1007/s00249-016-1171-9

Zhan, L., and Li, J. (2018). The role of TRPV4 in fibrosis. *Gene*. 642, 1–8. doi:10.1016/j.gene.2017.10.067

Zhang, L. P., Ma, F., Abshire, S. M., and Westlund, K. N. (2013). Prolonged high fat/alcohol exposure increases TRPV4 and its functional responses in pancreatic stellate cells. *Am. J. Physiol. – Regul. Integr. Comp. Physiol*. 304, R702–R711. doi:10.1152/ajpregu.00296.2012

Zhang, R., Liu, Q., Peng, J., Wang, M., Li, T., Liu, J., et al. (2020). CXCL5 overexpression predicts a poor prognosis in pancreatic ductal adenocarcinoma and is correlated with immune cell infiltration. *J. Canc*. 11, 2371–2381. doi:10.7150/jca.40517

Zhao, H., Guo, E., Hu, T., Sun, Q., Wu, J., Lin, X., et al. (2016). KCNN4 and S100A14 act as predictors of recurrence in optimally debulked patients with serous ovarian cancer. *Oncotarget*. 7, 43924–43938. doi:10.18632/oncotarget.9721

Zhao, L. M., Wang, L. P., Wang, H. F., Ma, X. Z., Zhou, D. X., and Deng, X. L. (2015). The role of K_{Ca}3.1 channels in cardiac fibrosis induced by pressure overload in rats. *Pflugers Arch. Eur. J. Physiol*. 467, 2275–2285. doi:10.1007/s00424-015-1694-4

Zhao, L., Zhao, H., and Yan, H. (2018). Gene expression profiling of 1200 pancreatic ductal adenocarcinoma reveals novel subtypes. *BMC Canc*. 18, 603. doi:10.1186/s12885-018-4546-8

Zheng, W., Thorne, N., and McKew, J. C. (2013). Phenotypic screens as a renewed approach for drug discovery. *Drug Discov. Today*. 18, 1067–1073. doi:10.1016/j.drudis.2013.07.001

Zhi, D., Zhao, X., Dong, M., and Yan, C. (2017). miR-493 inhibits proliferation and invasion in pancreatic cancer cells and inversely regulated hERG1 expression. *Oncol. Lett*. 14, 7398–7404. doi:10.3892/ol.2017.7178

Zhou, B., Irwanto, A., Guo, Y. M., Bei, J. X., Wu, Q., Chen, G., et al. (2012). Exome sequencing and digital PCR analyses reveal novel mutated genes related to the metastasis of pancreatic ductal adenocarcinoma. *Cancer Biol. Ther*. 13, 871–879. doi:10.4161/cbt.20839

Zhu, S., Zhou, H. Y., Deng, S. C., Deng, S. J., He, C., Li, X., et al. (2017). ASIC1 and ASIC3 contribute to acidity-induced EMT of pancreatic cancer through activating Ca2+/RhoA pathway. *Cell Death Dis*. 8, e2806. doi:10.1038/cddis.2017.189

Zhu, Y., Knolhoff, B. L., Meyer, M. A., Nywening, T. M., West, B. L., Luo, J., et al. (2014). CSF1/CSF1R blockade reprograms tumor-infiltrating macrophages and improves response to T-cell checkpoint immunotherapy in pancreatic cancer models. *Cancer Res*. 74, 5057–5069. doi:10.1158/0008-5472.CAN-13-3723

Zhuang, L., Peng, J.-B., Tou, L., Takanaga, H., Adam, R. M., Hediger, M. A., et al. (2002). Calcium-selective ion channel, CaT1, is apically localized in gastrointestinal tract epithelia and is aberrantly expressed in human malignancies. *Lab. Invest*. 82, 1755–1764. doi:10.1097/01.lab.0000043910.41414.e7

Zsiros, E., Kis-Toth, K., Hajdu, P., Gaspar, R., Bielanska, J., Felipe, A., et al. (2009). Developmental switch of the expression of ion channels in human dendritic cells. *J. Immunol*. 183, 4483–4492. doi:10.4049/jimmunol.0803003

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 Hofschröer, Najder, Rugi, Bouazzi, Cozzolino, Arcangeli, Panyi and Schwab. 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