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Organoids for drug discovery

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Organoids for drug discovery

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Editorial: Organoids for drug discovery

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organoids, iPSCs, drug screening, drug response prediction, drug efficacy and toxicity

Editorial on the Research Topic
Organoids for drug discovery

The term "Organoid", coined by Hans Clevers in 2009, refers to structures that closely mimic the architecture and function of specific organs. He focuses on elucidating the role of Wnt signaling in tissue homeostasis and oncogenesis. The discovery of Lgr5 as a marker of Wnt-stem cells in tissue led to the development of a technology that generates epithelial organoids from single stem cells.

Organoids retain key characteristics of the original tissue, making them suitable for studying physiology and pathology of diseases. (Patient derived organoids, PDO), which function as avatars, can also predict the most effective drugs for individual patients. Organoids models represent valuable approaches for studying cancer development and drug resistance, and their *in vitro* culture allows extensive manipulations, such as genetic modification. Researchers have constructed organoids of specific lineages, including brain, liver, kidney, lung, intestinal, and skin, using human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs). Organoids derived from hESCs or iPSCs recapitulate numerous features, such as high structural complexity, size, and organization.

However, most organoids lack the stromal, immune, neural, and vascular endothelial cell, limiting their utility in disease modeling. To model whole-body physiology and systemic diseases, multi-organ interaction chip with recirculating vascular flow and real-time monitoring system are required. Therefore, organoids models incorporating these key components represent an emerging platform with significant potential for evaluation of new drug efficacy and toxicity.

On 10 April 2025, the FDA announced plan to phase out animal testing, replaced using (New Approach Methodologies, NAM), including (Artificial Intelligence, AI) computational models and organoids based efficacy and toxicity testing. Al, particularly deep Learning models, can now design completely novel molecular structures that have never been synthesized and predict the strength of drug-target interactions as well as potential toxicity. The integrating of AI, organoids, and computational biology has emerged as a transformative approach for exploring novel therapeutic strategies and providing human-relevant toxicity data.

This Research Topic aims to translate basic scientific discoveries into clinical applications and highlight new researches in the field of organoids. The included studies cover the major areas of growing interest in organoids research. Shen et al. developed an iPSC derived kidney organoid exhibiting renal tubular and glomerular

structures and expressing specific kidney markers. This kidney organoid based prediction system was used to assess the protective effects of celastrol against cisplatin-induced nephrotoxicity. Wang et al. presented the strengths and weaknesses with the utilization of the ECM in kidney organoid culture. To explore the utility of organoid models in elucidating the reproductive complications of neurodrug exposure, Mariam et al. reviewed the principles of organoid models, emphasizing their ability to recapitulate neurodevelopmental processes and simulate drug-induced toxicity in a controlled environment. Xu et al. summarized organoid models developed for studying the mechanisms of diabetes and its complications, as well as for drug screening. Zou et al. introduced the definition and advantages of organoids and described their application in benign and malignant liver and biliary tract diseases, drug research, and regenerative medicine. In recent years, the application of PDO in drug detection and screening has rapidly expanded. Zhou et al. reviewed the application of colorectal organoid technology in basic methods and explores the pathogenesis of and personalized treatment of various colorectal diseases. Shen et al.summarized the use of organoids in modeling, drug efficacy assessment, and drug response prediction for ovarian, endometrial, and cervical cancers, offering valuable options for gynecological oncology patients.

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The reprotoxic adverse side effects of neurogenic and neuroprotective drugs: current use of human organoid modeling as a potential alternative to preclinical models

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The management of neurological disorders heavily relies on neurotherapeutic drugs, but notable concerns exist regarding their possible negative effects on reproductive health. Traditional preclinical models often fail to accurately predict reprotoxicity, highlighting the need for more physiologically relevant systems. Organoid models represent a promising approach for concurrently studying neurotoxicity and reprotoxicity, providing insights into the complex interplay between neurotherapeutic drugs and reproductive systems. Herein, we have examined the molecular mechanisms underlying neurotherapeutic drug-induced reprotoxicity and discussed experimental findings from case studies. Additionally, we explore the utility of organoid models in elucidating the reproductive complications of neurodrug exposure. Have discussed the principles of organoid models, highlighting their ability to recapitulate neurodevelopmental processes and simulate drug-induced toxicity in a controlled environment. Challenges and future perspectives in the field have been addressed with a focus on advancing organoid technologies to improve reprotoxicity assessment and enhance drug safety screening. This review underscores the importance of organoid models in unraveling the complex relationship between neurotherapeutic drugs and reproductive health.

KEYWORDS

drug-induced reprotoxicity, organoid model, neurotherapeutic drug, side effect, toxicicity

1 Introduction

Drug-induced toxicity poses a significant challenge in drug research and development, often leading to failures in clinical trials and subsequent drug withdrawals (Vo et al., 2019). Reproductive toxicity, encompassing both reproductive and developmental toxicities, substantially contributes to drug withdrawal, accounting for approximately 3%

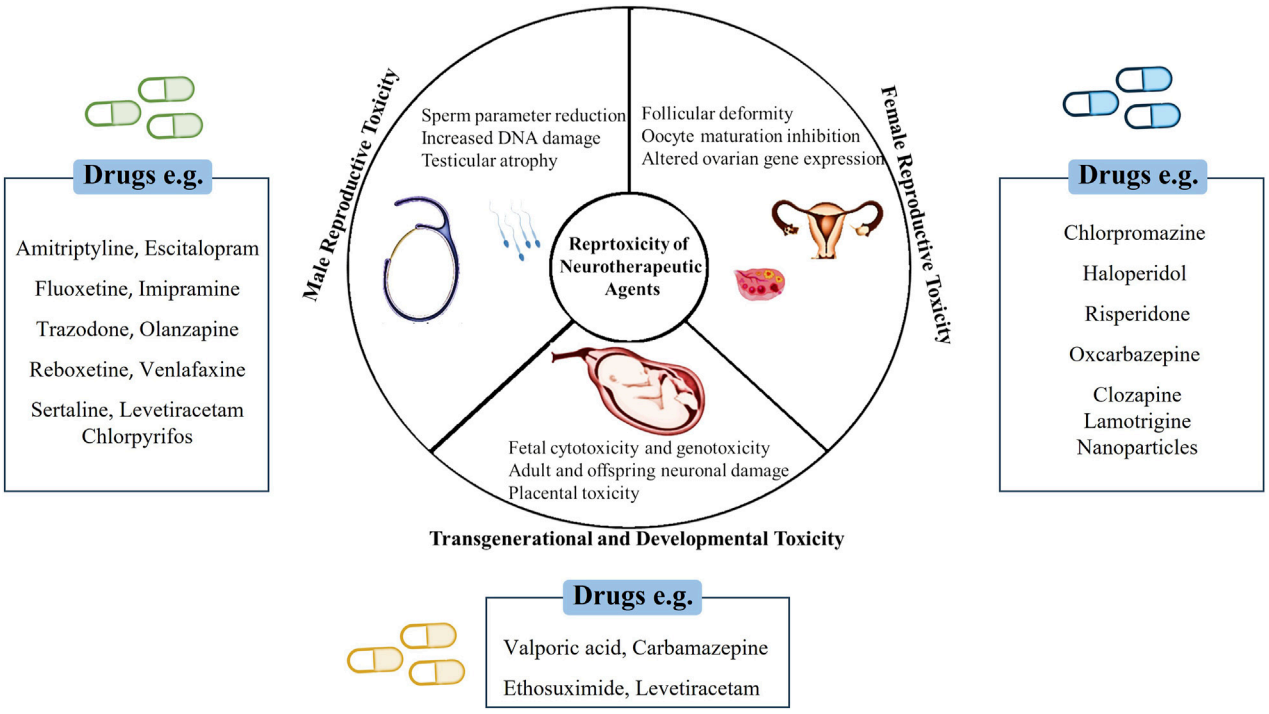

FIGURE 1
Schematic representation of neurotherapeutic agents' impact on various reproductive health.

and >10% of drug discontinuations and preclinical toxicology-related attrition, respectively (Siramshetty et al., 2016). To mitigate such risks, early assessment of the toxic properties of chemical compounds is paramount in drug development to mitigate such risks (Brannen et al., 2017). In reprotoxicity assessments, the impairment of male and female reproductive capacities and induction of nongenetic harmful effects on offspring are evaluated (Piparo, 2010) (Figure 1). However, traditional experiments for assessing chemical toxicity profiles, especially in animal models, are costly and time-consuming, with toxicity tests in animal models accounting for a significant proportion of compliance-related testing costs. Furthermore, the results of animal-based reprotoxicity tests may not always accurately predict human responses, adding complexity to toxicity endpoint assessments (Höfer et al., 2004). Additionally, discerning whether a compound directly affects reproduction or causes systemic toxicity that indirectly impacts reproductive systems further complicates the interpretation of experimental data. Consequently, the use of animal experiments alone might not fully reveal human responses to new drugs or provide reliable risk assessments, necessitating alternative approaches for toxicity assessment.

This review thoroughly examined neurotherapeutic drug-induced reprotoxicity in organoid modeling. The review first focused on stem cell applications for testing, exploring their potential and limitations. Then, it delved into the complex relationship between neurotherapeutic drugs and reproductive health, highlighting reprotoxic adverse effects. The second section offered a comprehensive analysis of the impact of neurotherapeutic drugs on reproductive health. Lastly, it showed future directions to improve technology fidelity and practical applications.

2 A brief history of organoids

Three-dimensional (3D) culture systems are generated using suspension culture to avoid direct contact with the plastic dish. This can be achieved through scaffold or scaffold-free methods. Scaffolds composed of biological or synthetic hydrogels mimic the natural extracellular matrix (ECM), and Matrigel® is the most prevalent matrix. Matrigel, a complex protein mixture derived from Engelbreth–Holm–Swarm (EHS) mouse sarcoma cells, contains adhesive proteins such as collagen, entactin, laminin, and heparin sulfate proteoglycans, providing structural support and ECM cues to cells. In scaffold-free methods, cells are cultured in droplets of defined medium suspended from a plate by gravity and surface tension (Unbekandt and Davies, 2010). Alternatively, 3D organoid structures can be formed using the air–liquid interface method, in which cells are cultured on a basal layer of fibroblasts or Matrigel initially submerged in a medium. As the medium evaporates, the upper cell layers are exposed to air, promoting polarization and differentiation (Kalabis et al., 2012).

The idea of *in vitro* regeneration of organisms traces back to 1907, when Henry Van Peters Wilson demonstrated that dissociated sponge cells could self-organize and regenerate an entire organism (Wilson, 1907). Subsequently, experiments involving cell dissociation and reaggregation in the mid-20th century resulted in the generation of various organs from dissociated amphibian pronephros (Holtfreter, 1943) and chick embryos (Weiss and Taylor, 1960). In 1964, Malcolm Steinberg proposed the differential adhesion hypothesis, suggesting that cell sorting and rearrangement could be explained by thermodynamics mediated by varying surface adhesion (Locke, 2012). After the isolation and establishment of pluripotent stem cells (PSCs) from mouse

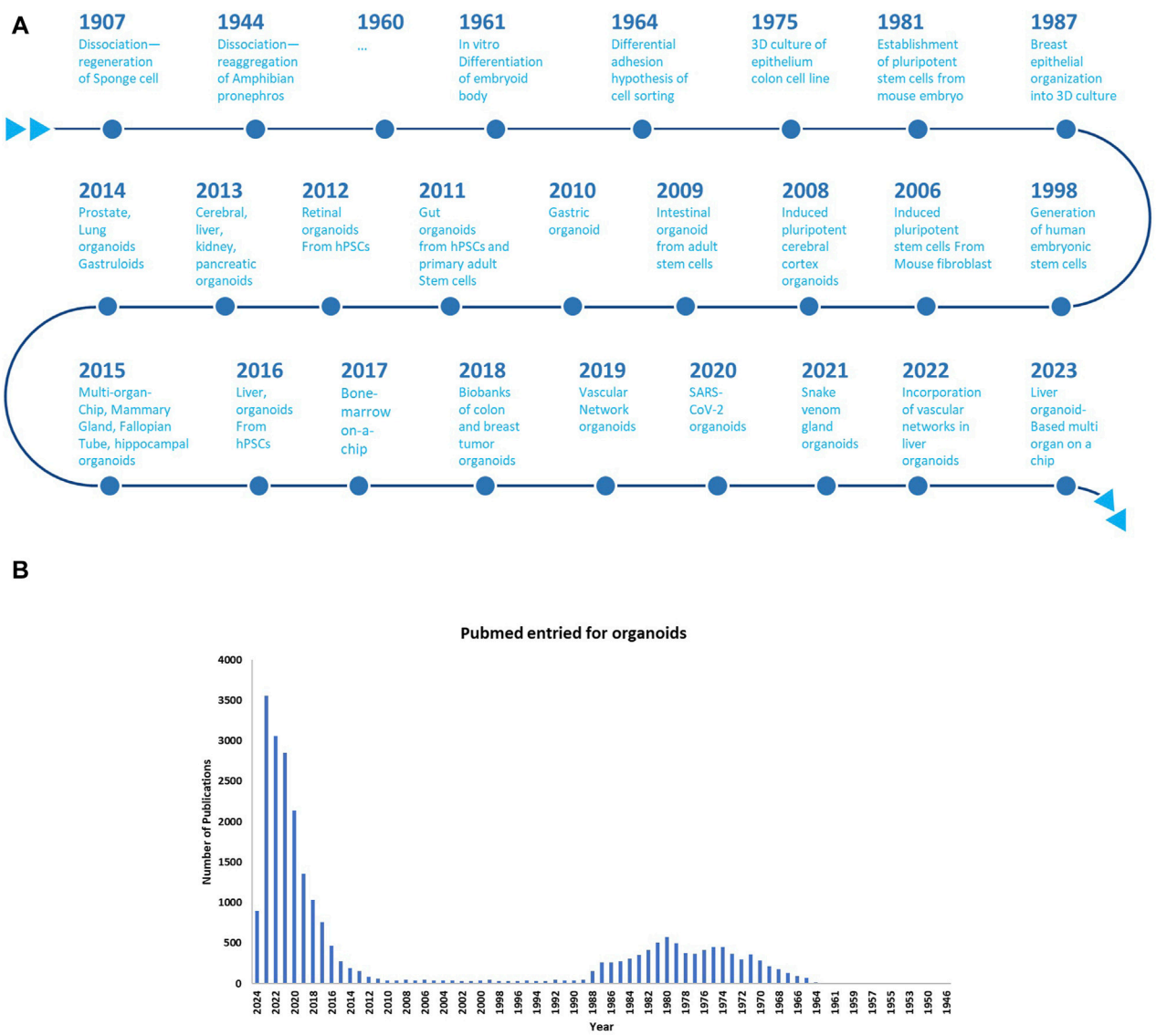

FIGURE 2
(A) Chronological evolution of three-dimensional (3D) organoid systems; **(B)** Increasing adoption of 3D Organoid Cultures in biological research: A PubMed Analysis till the year 2024.

embryos in 1981 (Evans, 1981; Martin, 1981) and human embryonic stem cells (ESCs) in 1998 (Thomson et al., 1998; Thomson et al., 1998), significant advancements were noted in stem cell research. Subsequently, induced pluripotent stem cells (iPSCs) were developed by reprogramming mouse and human fibroblasts, leading to a transformative impact on stem cell and organoid studies (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007).

In 1987, significant efforts were made in enhancing cell culture conditions to mimic the *in vivo* microenvironment. A previous study revealed that breast epithelial cells could generate 3D ducts and lumina when cultured on EHS ECM extract, enabling the production and secretion of milk protein; this phenomenon cannot be achieved in traditional two-dimensional (2D) culture (Li et al., 1987). Another study demonstrated that alveolar type II epithelial cells retain their specialized functions when cultured on ECM matrix, underscoring the crucial role of cell-matrix interactions in tissue homeostasis and differentiation (Shannon et al., 1987). The transition from 2D to 3D organoid culture was exemplified by the creation of cerebral cortex tissue from ESCs using the 3D aggregation culture technique (Eiraku et al., 2008). A groundbreaking study in 2009 demonstrated that adult intestinal stem cells expressing single leucine-rich repeat–containing G protein-coupled receptor five could form 3D intestinal organoids in Matrigel, organizing themselves into crypt–villus structures without requiring a mesenchymal niche (Sato et al., 2009) (Figure 2).

3 Organoid modeling techniques

Despite advancements in organogenesis research, fully replicating the human body *in vitro* remains challenging. Biological models like 2D/3D cell cultures and animal models

have been established to mimic human physiology but face limitations (Shariati et al., 2021). Conversely, stem cell technology holds immense promise for unraveling disease mechanisms and offering potential therapeutic interventions through human tissue modeling. In 1998, the advent of human blastocyst-derived ESCs, which can differentiate into all three germ layers during gastrulation, marked a significant milestone in this field. These cells have been extensively utilized in laboratories globally. The groundbreaking establishment of iPSCs from adult human fibroblasts in 2007, which was achieved through the expression of four key transcription factors (SOX-2, KLF4, OCT3/4, and c-MYC), further expanded the repertoire of PSC sources. This breakthrough enabled the long-term culture of stem cells and establishment of *in vitro* 3D structures, laying the foundation for precision medicine. In addition to ESCs and iPSCs, mesenchymal stem cells (MSCs) are also crucial in translational research due to their immune modulation and regenerative abilities (Thomson et al., 1998; Takahashi et al., 2007; Ballini et al., 2017).

3D cell culture models offer a more accurate *in vitro* environment than 2D cultures. Organoids from primary tissue or stem cells hold potential in regenerative medicine and personalized medicine (Hynds and Giangreco, 2013). However, they vary based on cell type. Pluripotent stem cells mimic fetal tissue but may face heterogeneity issues. Adult stem cells are well-defined but challenging to isolate, limiting their potential (Forbes et al., 2018; Tuveson and Clevers, 2019).

3.1 Organoids for the male reproductive system

The male reproductive system is intricate, encompassing the testes, ducts, glands, and penis. The testes are vital for sperm and testosterone production, while ducts aid in sperm transport and maturation. Seminal vesicles, prostate, and penis are crucial for reproduction. Dysfunction can cause issues like infertility and cancer. Studying in humans is challenging, but *in vitro* models like primary cultures and tissue explants are used. 3D organoids show promise for research (Patrício et al., 2023).

3.1.1 Testicular organoids

Testicular organoids, derived from healthy or diseased tissues, replicate testis structure and function. Baert et al. (2019) developed human testicular organoids by seeding adult and teen primary testicular cells into agar blocks, resulting in compact structures that can produce testosterone, form tight junctions, and support germ cell renewal. Other methods like alginate-based hydrogels and 3D bioprinting have also been used to generate testicular organoids, supporting spermatogenesis and Leydig cell functionality Matrigel®-based testicular organoid cultures in rodents, pioneered by Alves-Lopes et al., replicate *in vivo* testis features. They utilized a Matrigel gradient system for co-culturing Sertoli and germ cells. Typically, testicular organoid techniques involve direct cell-Matrigel mixing or a medium-Matrigel blend (Pendergraft et al., 2017; Yuan et al., 2020). In their study, a novel three-layer gradient system was developed by optimizing culture conditions, including the number of cells and concentration of Matrigel. Spherical–tubular structures were assessed using Sox9, Ddx4, and Scp3 as markers. Positive Ddx4 and Scp3 staining indicated the presence of germ cells, suggesting spermatogenesis potential. Sertoli cells, which are crucial for spermatogenesis, were positive for Scp3. Zo-1 expression suggested a potential blood–testis barrier (BTB), although this finding requires validation. Ki67 staining revealed the presence of proliferating cells (Wu et al., 2022).

These organoids exhibited a functional blood-testis barrier and maintained undifferentiated germ cells for prolonged periods (Alves-Lopes et al., 2017). Testicular organoids from porcine cells in collagen hydrogel mimic native testis architecture. 3D printing and microfluidic systems improve organoid fidelity (Vermeulen et al., 2019). Innovative techniques such as 3D printing and microfluidic systems have also been employed to enhance the fidelity of testicular organoids. Baert's group utilized 3D-printed scaffolds to develop structurally compartmentalized organoids from mouse testicular cells, promoting tubulogenesis and supporting germ cell differentiation (Richer et al., 2021). Testicular organoids offer vast potential for studying male reproductive health, including spermatogenesis and hormone regulation. They provide avenues for disease modeling, drug testing, and personalized medicine in male infertility (Patrício et al., 2023).

Recently, Stopel et al. produced testis organoids from primary testicular cells of neonatal mice using transwell inserts. Our findings demonstrate that these organoids form tubule-like structures and exhibit cellular organization similar to that observed in live testicular tissue (Stopel et al., 2024).

3.1.2 Epididymis organoids

The epididymis, which is vital for sperm maturation, is segmented into the caput, corpus, and cauda in larger mammals, with an additional initial segment in rodents (Pinel et al., 2019). It features a single convoluted tubule with a pseudostratified epithelium containing principal, clear, basal, and halo cells (Pinel et al., 2019). The blood-epididymis barrier (BEB) regulates luminal content and protects spermatozoa. Epididymal transit enhances sperm motility and fertilization potential through the luminal microenvironment and motility-affecting molecules (Sullivan and Belleannée, 2017). Generation of epididymal organoids was initially attempted using spheroid cultures from single cells, with human epididymal cells forming spheres under 2D conditions. Acinus formation in cultured rat epididymal basal cells was dependent on fibroblast growth factor (FGF) and dihydrotestosterone (Mandon et al., 2015). Basal cells could differentiate into principal cells, indicating existence of stem cell (Mandon et al., 2015). Recent developments by Leir et al. and Pinel and Cyr involved human and rat epididymal basal cells, respectively, forming 3D cultures (Leir et al., 2020; Pinel and Cyr, 2021).

3.1.3 Organoids for prostate glands

The prostate gland, essential for sperm nourishment and transport, comprises luminal cells, basal cells, and rare neuroendocrine cells within a pseudostratified epithelium (Crowley and Shen, 2022). Prostatic fluid, containing zinc, citric acid, prostate-specific antigen (PSA), and choline, is pivotal for sperm liquefaction post-ejaculation, facilitated by PSA degrading Semenogelin I and II. Novel experimental models have emerged for studying prostate cancer (PCa) due to limitations in traditional cell

lines and 2D cultures for drug screening. PCa organoids, derived from various cell sources, recapitulate the tumor microenvironment, aiding in understanding tumor development, progression, and therapy response. Examples include CRPC-derived organoids predicting enzalutamide sensitivity based on genetic alterations. High-throughput imaging assays enhance drug response analysis in diverse PCa phenotypes. Organoids elucidate mechanisms of drug resistance, like dual loss of TP53 and PTEN conferring resistance to anti-androgens. They serve in drug development and testing, using co-culture models to study microenvironmental effects and metastasis. Challenges include experimental variability and biopsy sample representativeness, with ongoing optimization for improved clinical outcomes in advanced PCa (Conteduca et al., 2020; Elbadawy et al., 2020; Gleave et al., 2020; Choo et al., 2021; Dhimolea et al., 2021).

Notably, urethral complications, from injury or congenital issues, present treatment challenges. Advances in stem cell research, notably 3D bioprinting, offer solutions. Tissue-engineered urethral grafts, pioneered by Atala and refined by Raya-Rivera, show promise in treating pediatric patients with urethral defects (ATALA et al., 1999; Raya-Rivera et al., 2011). Kajbafzadeh et al. explored regenerative methods, showcasing cell sheet techniques' effectiveness in urethral reconstruction (Kajbafzadeh et al., 2017). Challenges persist in 3D bioprinting regarding implant mechanical strength and biocompatibility. Efforts in developing urethral organoids and cultivating corpus spongiosum structures concurrently hold promise for clinical advancements (Patrício et al., 2023).

3.2 Organoids for the female reproductive system

The female reproductive system comprises the ovaries, fallopian tubes, uterus, cervix, and vagina. This system responsible for gamet and sex hormone production and pregnancy. Female reproductive tissue organoids, including human endometrial organoids, effectively model endometrial physiology and pathology. Derived from diverse stem cell sources, they accurately mimic glandular structures and functions, responding to hormones and replicating conditions like endometriosis and cancer. Utilizing CD146+ mesenchymal stem cells for endometrial-like epithelium creation offers prospects for regenerative medicine and embryo implantation studies. Additionally, 3D stromal cell models enable research on decidualization and angiogenesis. Further exploration is needed to fully leverage endometrial organoids for understanding implantation challenges and early pregnancy failure (Hennes et al., 2019; Mittal et al., 2019; Zambuto et al., 2019; Cui et al., 2020; Wiwatpanit et al., 2020).

3.2.1 Vulva organoids

The vulva, comprising various structures such as the labia majora, labia minora, and clitoris, serves as the initial defense barrier for the female reproductive tract. Although no organoids have been derived directly from the vulva, insights can be obtained from skin organoid studies due to the similarity in epithelial composition. Organoid studies on the skin have revealed spatiotemporal aspects of epidermal development and facilitated the long-term expansion of keratinocytes, offering a potential model for studying gene alterations implicated in vulvar diseases and carcinogenesis. Sweat glands, crucial for microbial homeostasis, have been studied using organoid cultures, suggesting a possible avenue for developing vulvar sweat gland-derived organoids. Overall, vulva-derived organoids hold promise for understanding epithelial biology, microbiome interactions, and diseases like genital infections and vulvar cancers (Boonekamp et al., 2019; Diao et al., 2019).

3.2.2 Vaginal organoids

Research on vaginal development, primarily conducted in mice, has highlighted the intricate interplay between epithelial cells and the underlying stroma, influence of hormone receptor genes, and pivotal role of the Wnt/β-catenin pathway (Heremans et al., 2021). Recently, Ali et al. (2020) established a sophisticated 3D organoid culture system using mouse vaginal epithelial cells. This innovative model revealed the critical roles of Wnt and BMP signaling pathways in maintaining the stem cell niche within the vaginal epithelium. By meticulously controlling the culture conditions and manipulating key signaling molecules such as EGF, TGF-βR, and ROCK inhibitors, they expanded and sustained these organoids *in vitro* (Ali et al., 2020). Moreover, the identification of specific markers, such as AXIN2, provided insights into cellular hierarchy and lineage differentiation within the vaginal epithelium.

3.2.3 Cervix organoids

The cervix, which is vulnerable to human papillomavirus -induced cancer, lacks accurate modeling in 2D cultures. 3D organoids provide a physiologically relevant platform for studying cervical cancer mechanisms. Cervical organoids, derived from patient biopsies, express specific markers and exhibit differentiation, offering insight into cervical cancer development. They enable studies on pathways like Wnt signaling in tumor progression (Heremans et al., 2021). Moreover, cervical organoids offer potential applications in personalized medicine, allowing for the testing of patient-specific drug responses. Maru et al. (2020) successfully developed cervical organoids from patient-derived biopsies using a specific medium containing RSPO1, Noggin, EGF, ROCKi, and Jagged-1. Cervical organoids exhibited enhanced expression of SCJ markers compared to traditional cell lines and demonstrated differentiation into both endo- and ectocervical cell types. Chumduri et al. (2021) also created long-lasting endocervical-like organoids from patient samples, reliant on Wnt agonists RSPO1 and WNT3A, showing potential differentiation towards ectocervical characteristics (Chumduri et al., 2021). Maru et al. (2019a, b) developed cervical clear cell carcinoma organoids using established culture conditions. Xenografting these organoids in mice allows for more clinically relevant treatment efficacy evaluation (Maru et al., 2019a; Maru et al., 2019b).

3.2.4 Endometrial orgnaoids

The endometrium, which lines the uterus, sheds monthly under hormonal regulation. It consists of two layers: lamina basalis and lamina functionalis. The source of the endometrium remains controversial, and possible sources include stem and bone marrow-derived cells. Uncertainty exists regarding the hierarchy

of proposed stem cell candidates and their translation to humans. Studies have been conducted to understand endometrial regeneration signaling. Previous 3D culture attempts were limited by short lifespans and inadequate *in vivo* mimicry, indicating the need for more representative models. For instance, Iguchi et al. (1985) successfully cultured luminal mouse endometrial cells on collagen gel matrices in serum-free conditions, exhibiting characteristics akin to adenogenesis, despite their short-lived nature (Iguchi et al., 1985). Similarly, Rinehart et al. (1988) seeded 3D endometrial glands on Matrigel-coated plates, resulting in structures with apicobasal polarity and preserved intercellular connections, albeit spreading out into 2D monolayer colonies (Rinehart Jr et al., 1988). Previous efforts faced challenges like low serum requirements, limited long-term maintenance, and incomplete endometrial characteristics. Ongoing work aims to develop advanced 3D endometrial models. Recently, organoids were cultured successfully using a defined medium (Boretto et al., 2017; Turco et al., 2017; Haider et al., 2019). RSPO1 or CHIR99021 activation of Wnt/β-catenin signaling was essential for their development, reflecting Wnt's role in uterine gland formation. *In vivo* lineage tracing identified AXIN2+ cells as potential stem cell candidates in the mouse uterus (Syed et al., 2020). Human endometrial organoid development did not require exogenous WNT3A. Inhibition of BMP (Noggin) and TGF-β/Alk (A83-01) pathways was crucial, alongside EGF, FGF10, 17β-estradiol (E2), insulin (ITS), and inhibition of p38 MAPK, ROCK, and sirtuin. Hormone treatment replicated the menstrual cycle and early decidualization (Haider et al., 2019; Hennes et al., 2019; Bui et al., 2020; Cochrane et al., 2020; Syed et al., 2020). Trophoblast organoids were derived with minimal differences in medium. Marinić et al. (2020) derived endometrial gland organoids from the term placentas with slight modifications, showing hormone responsiveness and distinct molecular patterns (Marinić et al., 2020).

Importantly, adenomyosis and endometriosis involve ectopic endometrial tissue. Adenomyosis is within the uterine wall, while endometriosis involves tissue outside the uterus. Primate studies offer insights, but ethical concerns arise. Human-derived 3D organoids show promise for studying endometriosis (Heremans et al., 2021). Enriching organoid cultures with diverse cell types for studying adenomyosis and endometriosis is crucial. A reported co-culture model includes adenomyotic epithelial cells, stromal cells, and myocytes (Mehasseb et al., 2010). Although AXIN2+ cells were suggested as endometrial cancer (EC)-initiating cells in mice, the human counterpart remains unidentified (Syed et al., 2020). Patient-derived EC cell lines (e.g., Ishikawa, RL95-2) showed genomic stability but lacked intra-tumor heterogeneity (Van Nyen et al., 2018). Mouse xenograft models showed fair engraftment rates but struggled to replicate tumor microenvironments (Depreeuw et al., 2015). 3D culturing techniques, including spheroid cultures, revealed altered metabolism and drug susceptibility. Organoid development relied on key factors like RSPO1, EGF, and FGF2, with validated genomics showing mutations in ARID1A, CTNNB1, and PTEN (Boretto et al., 2019). Organoids were tested for sensitivity to chemotherapeutics and inhibitors targeting PI3K and mTOR pathways (Pauli et al., 2017; Heremans et al., 2021). Boretto et al. (2019) showed that organoids can replicate different endometrial states, including cancerous ones like Lynch syndrome mutations. This lays the groundwork for advanced organoid models for co-culture systems, drug testing, and gene-editing studies (Boretto et al., 2019).

3.2.5 Fallopian tube organoids

Fallopian tube organoids derived from fallopian tube epithelial cells (FTECs) offer insights into infertility, tumor etiology, and drug effects (Chang et al., 2020). FTE organoids exhibit polarized columnar cells, tight junctions, and functional similarities to native tissue (Chang et al., 2020). They replicate mucosal fold architecture, express secretory markers, and respond to hormonal cues akin to the fallopian tube epithelium (Chang et al., 2020). These organoids present a valuable model for studying fallopian tube biology and pathology. Additionally, Fallopian tube organoids derived from FTECs or iPSCs accurately model fallopian tube biology and pathology, exhibiting distinct gene profiles and anatomical features compared to 2D cell lines. iPSC-derived organoids replicate fallopian tube anatomy through precise differentiation steps and expression of specific markers (Yucer et al., 2017). These organoids serve as disease models for conditions like chronic *chlamydia* infection (Kessler et al., 2019). However, challenges remain in long-term culture maintenance and achieving functional maturity for studying high-grade serous ovarian cancer (sHGSC) (Cui et al., 2020).

3.3 Embryonic and fetal organoids

Organoids have been developed from the embryonic and fetal stages. Trophoblast organoids derived from cytotrophoblasts (CTBs) model early placenta formation and placental diseases (Turco et al., 2018). Cultured in trophoblast organoid medium (TOM), CTB organoids closely mimic the morphology, differentiation ability, and gene expression patterns of human placental villi (Haider et al., 2018; Haider and Beristain, 2023). They demonstrate stemness, proliferation, and fusion characteristics akin to villous cytotrophoblasts (vCTBs), making them suitable for modeling implantation. While CTB organoids hold promise for disease modeling and investigating trophoblast invasion, further optimization is needed to enhance self-renewal, specificity, and differentiation (Turco et al., 2018). Additionally, human iPSCs produce trophoblast cystic structures resembling trophectoderm for implantation studies. Previous 3D models lacked true organoid characteristics (Wong et al., 2018; Cui et al., 2020).

Modeling the ovarian surface epithelium (OSE), the origin of most malignant ovarian tumors is crucial for exploring endometriosis etiology (Lawrenson et al., 2009). While 3D culturing of normal OSE remains unexplored for organoid construction, human oocytes have been mimicked in 3D structures derived from human embryonic stem cells (hESCs), showing meiotic entry and oocyte-like characteristics (Jung et al., 2017). These follicle-like cells (FLCs) express oocyte-specific markers, providing a platform for investigating human germ cell development and gene mechanisms, including noncoding RNAs (Jung et al., 2017; Cui et al., 2020).

Meanwhile, studying early human embryo development is challenging due to limited access to embryonic tissues. Pluripotent stem cells and surplus blastocysts offer insights, but

ethical constraints restrict their use (Haider and Beristain, 2023). Recent advances in stem cell-based blastocyst models, known as blastoids, resembling natural blastocysts, provide insights into early embryonic development. They exhibit spatial organization similar to epiblast, hypoblast, and trophectoderm-like cells, with efficiencies ranging from 2% to 80%. Differentiation protocols prioritize lineage expansion, resulting in blastoids resembling blastocyst morphology and implantation processes (Theunissen et al., 2014; Rostovskaya et al., 2019). Blastoids aid in studying embryogenesis, implantation, and early pregnancy. Co-culture with endometrial cells reveals intercellular communication's role in blastocyst attachment, with applications spanning developmental biology, fertility, and embryo safety (Kagawa et al., 2022).

Additionally, human placental research faces hurdles due to ethical constraints and limited tissue access. Trophoblasts, crucial for nutrient exchange, show species-specific developmental pathways, despite shared functions with rodents (Haider and Beristain, 2023). In 2018, 2D and 3D long-term regenerative trophoblast cultures were established from progenitor cytotrophoblasts (CTB) of first-trimester chorionic villi (<8 weeks gestation) (Haider et al., 2018; Okae et al., 2018). These cultures express trophoblast lineage genes, exhibit hypomethylation of the ELF5 promoter, and express chromosome 19 miRNA cluster micro RNAs. They can be perpetuated long-term with specific signaling conditions, fostering stem-like states and spontaneous fusion into hormone-producing multinucleated syncytiotrophoblast (SCT) in both 2D and 3D conditions. Removal of Wnt-activating factors prompts CTB differentiation into invasive extravillous trophoblasts (EVT). Trophoblast organoids represent a significant advancement in in vitro models, closely mirroring trophoblast cell lineage complexity observed *in vivo* (Haider et al., 2018). Single-cell transcriptomics confirms differentiation trajectories along extravillous and villous pathways (Shannon et al., 2022). Recent work addressed the limitations of blastoid models, including inverted syncytial structures, by utilizing suspension culture with gentle agitation. This approach produced organoids with properly oriented large syncytial structures that secrete high levels of SCT-associated factors (Yang et al., 2024). The choice of trophoblast progenitor source is crucial for trophoblast organoid design. While primary CTBs and TSC lines can both yield organoids, those from TSC lines may better resemble EVT progenitor-like cells transcriptionally and in surface marker expression (Sheridan et al., 2021). Moreover, TSC lines cultured in 2D or 3D exhibit detectable levels of Class I HLA-A/B, with 3D culture partially reducing their expression (Sheridan et al., 2021). Recent findings suggest that progenitors in hTSC line-derived organoids resemble a developmentally downstream state akin to column CTB (Shannon et al., 2022b). Though hTSC lines are favored for trophoblast studies, evaluating their merits and limitations is vital, particularly considering recent reports on their derivation from pluripotent stem cell (PSC) sources and induced TSCs (Karvas et al., 2022; Soncin et al., 2022; Tan et al., 2022). Trophoblast organoids aid in studying stem cell dynamics, highlighting YAP1 signaling's importance via inhibition and CRISPR-Cas9-mediated knockout experiments (Meinhardt et al., 2020). Additionally, trophoblast organoids have elucidated the role of TGFβ signaling in EVT development, showing that exogenous TGFβ impulse is necessary for EVT marker expression, while its inhibition results in pro-migratory/pro-invasive features (Haider et al., 2022). Moreover, trophoblast organoids have been utilized to study vertical viral infection routes and ensuing inflammatory responses in decidual cells. Human cytomegalovirus infection triggers a Type III interferon response in trophoblast organoids, offering protection to decidual cells (Yang et al., 2022). iPSC-derived TSC organoids have also shed light on ZIKA and SARS-CoV-2 virus interactions with trophoblasts (Karvas et al., 2022). However, it is important to note limitations such as the inverted nature of the organoid and expression of MHC class I ligands not typically seen in progenitor CTB or SCT, which may impact interpretations related to pathogen-host modeling.

Interestingly, 3D organoids are crucial for modeling embryonic development, especially for the pituitary gland and hypothalamus (Chukwurah et al., 2019). The SFEBq method enables the three-dimensional culturing of ES cells, promoting differentiation into ectodermal derivatives (Watanabe et al., 2005; Eiraku et al., 2008; Sasai et al., 2012). Utilizing SFEBq cultures, hypothalamic neurons were successfully induced from mouse ES cells (Wataya et al., 2008). Growth factor-free, chemically defined medium (gfCDM) supplemented with Sonic Hedgehog (SHH) optimally induces hypothalamic neuron differentiation (Suga, 2016). Crucially, specifying the rostral hypothalamic fate of mouse ES cells relied on removing exogenous growth factors rather than adding specific inductive signals (Chukwurah et al., 2019).

4 Neurotherapeutic drugs and reproductive health

Neurotherapeutic drugs play critical roles in managing neurological disorders, and their impact on reproductive health has been increasingly recognized. As depicted in Table 1, these drugs exert diverse effects on various aspects of reproductive function, including sperm quality, hormone levels, and embryonic development. Understanding these interactions is essential for optimizing treatment strategies and minimizing potential adverse effects on reproductive outcomes.

4.1 Antidepressants

Depression affects approximately 300 million people worldwide and is a significant global health issue (Sołek et al., 2021b). Antidepressants, initially developed in the 1950s, constitute the primary treatment for depression, and they target neurotransmitter imbalances in the brain (Sartorius et al., 2007). However, long-term use of antidepressants, often necessary for full therapeutic benefits, can lead to sexual dysfunctions, affecting patients' self-esteem and treatment compliance ((Montejo et al., 2019). These dysfunctions include reduced sexual desire, arousal difficulties, and orgasmic dysfunction (Higgins et al., 2010). The relationship between depression, pharmacotherapy, and sexual dysfunction is complex and lacks clear clinical guidelines (Hellstrom, 2008; Segraves and Balon, 2014). Antidepressants modulate neurotransmitters like serotonin, norepinephrine, and dopamine, influencing the sexual response cycle, and potentially explaining their effects on sexual function (Graf et al., 2019). Further

TABLE 1 Neurotherapeutic agents' adverse impacts on reproductive systems and fetal development.

Therapeutic class	Drug	Mechanisms	Example toxicities	Study type	References
Antidepressant	-Amitriptyline	-Formation of micronuclei	Mouse-derived spermatogonia and Spermatocyte	*In vitro*	Solek, et al. (2021)
	-Escitalopram	-Increase in telomeric binding factor (TRF1/TRF2) protein expression			
	-Fluoxetine	-Initiation of apoptotic cell death			
	-Imipramine	-Varied toxicity on mouse spermatogenic cells			
	-Trazodone	-Decreased sperm concentration, motility, and normal morphology; increased sperm DNA damage			
	-Olanzapine	-Elevated serum levels of FSH, LH, and testosterone			
	-Reboxetine	-Augmented oxidative stress			
	-Venlafaxine				
Selective serotonin reuptake inhibitors	Fluoxetine	Maladaptive offspring production	flea *Daphnia magna*	*In vivo*	Campos, et al. (2016)
	Sertaline	-Increased sperm DNA damage and induced histopathological lesions	Male rat		Atli, et al. (2017)
		-Abnormal sperm morphology and increased malondialdehyde (MDA) degeneration in cellular-tubular structures			
		-Elevated serum LH and testosterone levels			
		-Enhanced oxidative stress (OS)			
		Testicular toxicity			
	Citalopram	-Decrease in sperm motility	Men	Clinical	Safarinejad, 2008
	Escitalopram	-Abnormal sperm DNA fragmentation			Koyuncu, et al., 2011
	Fluoxetine				Safarinejad, 2008; Atli, et al., 2017
	Paroxetine				Tanrikut, et al., 2010
Antipsychotics	Chlorpromazine	-Increased activity of caspases-3, -8, and -9			Elmorsy, et al., 2017
	Haloperidol	-Elevated ROS (Reactive Oxygen Species) production	Female rat	*In vivo*	
	Risperidone	-Decreased total intracellular glutathione levels	Rat's ovarian theca interstitial cells	*In vitro*	
	Clozapine	-Heightened lipid peroxidation (LPO)			
	Olanzapine	-Elevated prolactin levels	Man	Clinical	Konarzewska, et al., 2009
	Risperidone	-Reproductive hormone disorders identified			
		Sexual dysfunction observed			
Antiepileptics		-Initially upregulates Aldh1a2	Zebrafish embryo toxicity	*In vivo*	Beker van Woudenberg, et al., 2014
	-Valporic acid	-Subsequently downregulates Cyp26a1			
	-Carbamazepine	-Suggests a teratogenic mechanism			
	-Ethosuximide	- Hatching and Motor Activity			
	-Levetiracetam	-Pericardial Edema			
		-Motor Activity Suppression			
	Valporate	-Elevated serum concentrations of Testosterone	Women	Clinical	Morrell, et al., 2002; Asif, 2017

(Continued on following page)

TABLE 1 (*Continued*) Neurotherapeutic agents' adverse impacts on reproductive systems and fetal development.

Therapeutic class	Drug	Mechanisms	Example toxicities	Study type	References
		-High prevalence of Menstrual Disorders, PCO, and PCOS			
		-Increased concentration of Androgens	Man		Mikkonen, et al., 2004; Asif, 2017
		-Abnormalities in sperm quality			
		-Reduced testicular volume			
	Oxcarbazepine	-Higher prevalence of PCO	Women		Mikkonen, et al., 2004; Asif, 2017
		-Higher serum concentrations of Androgens			
		-Higher serum concentrations of DHEAS			
		-Increased frequency of morphologically abnormal sperm	Man		Artama, et al., 2004; Asif, 2017
		-Lower serum testosterone levels			
	Carbamazepine	-Menstrual disorders linked to reduced bioactive E2 levels, indicated by altered E2/SHBG ratio	Women		Isojarvi (2001)
		-Reduced sperm concentration and high frequency of poorly motile sperm	Man		Perucca (2004)
		Induces hepatic P450 enzymes, elevating SHBG levels and lowering bioactive androgens			
	Lamotrigine	-High incidence of abortion and embryo lethality	Female rat	*In vivo*	Padmanabhan, et al., 2003; Hejazi and Taghdisi, 2019
		Congenital malformations and intrauterine growth retardation			
		-Decreased pup birth rate			
		-Significant reduction in body weight	Male rat		Daoud, et al., 2004
	Vigabatrin	-Reduction in the weight of testes, epididymis, seminal vesicles, ventral prostate, and vas deferens			
	Gabapentin				
	Levetiracetam	Dose-dependent decreases in sperm concentration, motility, and normal morphology	Male rat	*In vivo*	Baysal, et al. (2017)
		Increased sperm DNA damage observed			
		Alterations in oxidative stress markers indicating tissue damage			
Anti-cholinesterase	Dimethoate		Male Mice	*In vivo*	Verma and Mohanty, 2009
	Chlorpyrifos	-Reduced epididymal and testicular sperm counts			Joshi et al., 2007; Mor and Soreq, 2011
		-Decreased serum testosterone concentration			
		Pathological degeneration of seminiferous tubules			
		-Reduction in testicular glycogen and sialic acid content			
		-Increased cholesterol and protein content, dose-dependent			
	Malathion	-Increased testicular acid phosphatase activities			Choudhary et al., 2008; Mor and Soreq, 2011
		-Inhibition of testosterone secretion by Leydig cells			

(Continued on following page)

TABLE 1 (*Continued*) Neurotherapeutic agents' adverse impacts on reproductive systems and fetal development.

Therapeutic class	Drug	Mechanisms	Example toxicities	Study type	References
Opioid	Methadone	-Considerable increase in oxidative stress levels	Male rats	*In vivo*	Haddadi, et al., 2020
	Buprenorphine	Loss of gonadotropin hormones observed			
		Changes detected in sperm parameters (Haddadi, Ai et al., 2020)			
	Morphine	-Testis atrophy observed			Bu, et al., 2011; Ghowsi and Yousofvand 2015; Moinaddini, et al., 2023
		Reduction in the number of germ cells			
		Weight loss in the testis, prostate, and seminal vesicles			
		Associated with morphine dependence			
Nanoparticles	Silver nitrate NPs	-Follicular growth deformities, oocyte maturation inhibition	Female rat	*In vivo*	Charehsaz, et al., 2016
		-Damaged neurons in hippocampal regions of adult and offspring rats			
	Titanium dioxide NPs	Concentration-dependent alteration in ovarian gene expressions	Female Mice	*In vivo*	Karimipour, et al. (2018)
	Aluminum oxide NPs	-Placental toxicities -Cytotoxicity and genotoxicity	*Ex vivo* Chinese hamster ovary cell line	*In vitro*	Di Virgilio, et al., 2010

research into direct interactions between antidepressants and reproductive cells is necessary to better understand and manage their side effects (ST et al., 2006; Solek et al., 2021).

4.1.1 The reprotoxic side effects of treating depression

Most authors agree that Antidepressants and neuroleptic drugs are associated with sexual dysfunction, potentially due to their reproductive toxicity, impacting germ cell development, embryonic cell apoptosis, and fertility (Spiller et al., 2009; Kanatsu-Shinohara et al., 2010; Sato et al., 2011; Sato et al., 2012). One study demonstrated decreased ATP production in spermatogenic cells treated with amitriptyline hydrochloride, escitalopram, fluoxetine hydrochloride, imipramine hydrochloride, mirtazapine, olanzapine, reboxetine, and venlafaxine hydrochloride after 48 and 96 h, suggesting potential impairment of mitochondrial function (Sołek et al., 2021a).

Under stress, cells often arrest their cycle to facilitate repairing damage, resulting in changes to the cell cycle profile. Antidepressant-induced oxidative stress leads to increased DNA fragmentation and micronuclei formation, potentially due to reduced mitochondrial potential and irreversible damage (Aitken et al., 2014). Notably, antidepressants may influence the cell cycle, potentially inducing apoptotic cell death via various pathways. In particular, changes in cyclin D2 activity, governing the G1 to S phase transition, were observed, alongside the activation of proteins involved in cell cycle regulation (Biber et al., 2018). The activation of proteins (p16, p21, p27, p53) suggests their role in regulating the cell cycle via CDK inhibition (p16), DNA replication initiation (p21), G1/S phase transition (p27), and cell division (p53) (Roshdy and Fyiad, 2010; Mao et al., 2011; Battal et al., 2013; Biber et al., 2018). This activation hints at DNA damage repair and adaptive responses. Dysregulation of the cell cycle, crucial for spermatogenesis, may impair germ cell development, induce embryonic cell apoptosis, and reduce fertility (Spiller et al., 2009; Kanatsu-Shinohara et al., 2010; Sato et al., 2011; Sato et al., 2012). Antidepressants influence cell differentiation and proliferation through glucocorticoid receptor phosphorylation, upregulating p27 and p57, and induce cell cycle arrest in non-spermatogenic cells by inhibiting ERK1/2 kinase phosphorylation, altering gene expression in the p21/p53 pathway (Krishnan et al., 2008; Pechnick et al., 2011).

Disturbances in mitotic and meiotic processes, chromosomal aberrations, and reductions in sperm count, motility, and morphology are consistently observed across experimental sets (Alzahrani, 2012; Hassanane et al., 2012). Furthermore, the crucial protein NuMa, responsible for organizing and stabilizing the mitotic spindle apparatus, may be affected, leading to abnormal mitotic spindle formation after exposure to antidepressant drugs (Bhattacharya et al., 2013). Additionally, the interaction of cytotoxic drugs with tubulin subunits may disrupt microtubule polymerization and depolymerization, thereby impairing the function of the mitotic spindle. These highlight the complex impact of antidepressant treatment on reproductive health. Telomere dysfunction leads to genomic instability, apoptosis, or cellular senescence (Picco et al., 2016). Research has indicated a relationship between the levels of TRF1 and TRF2 proteins, expression of p53 and MAPK kinase, and the induction of apoptosis. Cell lines treated with antidepressants showed increased TRF1 and TRF2 synthesis. While some studies provide evidence of telomere length reduction in depressive disorder patients (Ridout et al., 2016), and changes in the amount of mitochondrial DNA (mtDNA) or telomere length due to stress and depression (Powell et al., 2018), there is insufficient data on TRF1 and TRF2 expression changes after antidepressant treatment.

In the final phase, cell death mechanisms are activated, marked by increased cleavage of caspase three and reduced synthesis of Bcl-2. The interplay of proteins within the Bcl-2 family significantly influences cell fate, determining whether cells survive or undergo apoptosis (Djordjevic et al., 2012). Additionally, the Bcl-2 family mediates the intrinsic pathway of apoptosis, wherein mitochondria play a significant role. Some findings suggest that patients with depressive disorders exhibit low expression of anti-apoptotic Bcl-2, which is increased with antidepressant treatment (Sakr et al., 2013; Khaksar et al., 2017). Preclinical studies have also demonstrated that antidepressants elevate Bcl-2 levels, offering protection against apoptotic cell death through interaction with mitochondrial voltage-dependent anion channels. Apoptosis is common in the testis as a safeguard mechanism for eliminating defective germ cells (Shukla et al., 2012) which is exacerbated by antidepressant usage inducing DNA fragmentation and lipid peroxidation, leading to cellular damage (Atli et al., 2017b). While confirmation in complex *in vivo* models is necessary due to testicular tissue complexity, these findings offer valuable insights into antidepressant-induced reproductive toxicity (Mytych et al., 2017).

4.1.2 Selective serotonin reuptake inhibitors

Selective serotonin reuptake inhibitors (SSRIs), commonly used to treat depression, raised concerns for endocrine-disruption. Human studies suggest developmental toxicity, reversible sexual dysfunction, and sperm DNA damage. In aquatic environments, fluoxetine, an SSRI component, acts as a neuroendocrine disruptor, affecting fish fertility and behavior. *Daphnia magna*' studies reveal SSRIs alter reproductive responses and offspring size, reversed by cyproheptadine. These findings highlight serotonin's role in reproductive regulation, necessitating further research (Campos et al., 2016).

In arthropods, serotonin neurons regulate vital processes like oogenesis, growth, and behavior. Yet, understanding of non-decapod crustaceans like Daphnia is limited. Evidence suggests serotonergic neurons influence growth and reproduction in Daphnia, inferred from SSRIs' effects and the presence of serotonin biosynthesis enzymes in their genome. Neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) damages serotonergic neurons, leading to oxidative damage, hypoxia, and neurotoxicity (Lamichhane et al., 2014; Campos et al., 2016; Nation Sr, 2022).

Daphnia's stress response involves adjusting reproductive investment to maximize fitness. Serotonergic interneurons in the brain regulate this process, as evidenced by SSRIs' effects. Fluoxetine increases serotonin-immunoreactivity under low food conditions, mimicking "optimum" reproductive conditions, while 5,7-DHT reduces it, aligning with reduced reproduction. These findings highlight serotonin's crucial role in Daphnia's reproductive investment regulation, revealing adaptive mechanisms in varied food environments (Gorbi et al., 2011; Gaukler et al., 2015).

Infertility affects 15% of couples, with male factors contributing to 30%–50% of cases. Factors like varicocele, infections, endocrine disorders, obesity, radiation, and drug use, including SSRIs, can impact male fertility by affecting sperm parameters and hormonal balance (Atli et al., 2017a).

SSRIs like sertraline, fluoxetine, and trazodone can impact male fertility due to their effects on sperm parameters and hormonal balance. SSRIs commonly prescribed for depression, such as sertraline, fluoxetine, and trazodone, are associated with sexual side effects, potentially affecting sperm count and morphology. For instance, Trazodone an antidepressant with anxiolytic and sleep-inducing effects, is widely used for insomnia. While clinical studies have linked SSRIs to decreased sperm quality, trazodone's reproductive toxicity remains underexplored, particularly in males of reproductive age (Nørr et al., 2016). A study found that TRZ administration reduced sperm concentration in male rats, motility, and normal morphology while increasing sperm DNA damage and testicular degeneration. Elevated serum levels of FSH, LH, and testosterone, along with oxidative stress in testicular tissue, were also observed (Ilgın et al., 2018). These hormonal changes were associated with decreased sperm quality and testicular degeneration. Additionally, trazodone exposure led to oxidative stress, as indicated by elevated levels of malondialdehyde (MDA) in testicular tissue, reflecting lipid peroxidation (Rahal et al., 2014; Sabeti et al., 2016; Ilgın et al., 2018). Clinical research on patients undergoing TRZ treatment is crucial for identifying potential reproductive toxicity, emphasizing the importance of monitoring sperm parameters before, during, and after TRZ therapy.

4.1.3 Lithium

Lithium salts are commonly used to treat major depressive disorders, with exposure occurring through various sources such as drinking water, food, and the environment. While low levels can alleviate depression, prolonged therapeutic doses may lead to complications affecting the renal, nervous, thyroid, and circulatory systems. Furthermore, Li + exposure can result in teratogenic effects and sterility (Aral and Vecchio-Sadus, 2008; Ommati et al., 2021).

As evidence, One study aimed to explore Li^+'s adverse effects on testicular tissue, spermatogenesis, and hormones using *in vitro* and *in vivo* models. *In vitro*, Leydig cells were cultured with Li^+ at escalating concentrations (0–100 ppm), while mice were given Li+ in drinking water (0–100 ppm) for 5 weeks *in vivo* (Ommati et al., 2021). Testicular and sperm samples were analyzed. Notably, oxidative stress has been implicated in testis and sperm injury in animals treated with Li^+. Li^+ affects testosterone biosynthesis *in vivo* and *in vitro*. Importantly, mitochondrial impairment plays a critical role in sperm and Leydig cells abnormality, and testis injury with Li^+-exposure (Folgerø et al., 1993). Li^+ induces decreased sperm motility via mitochondrial impairment and reduced ATP levels (Yousefsani et al., 2020). Li^+ adversely affects the reproductive system, with oxidative stress playing a crucial role. Sperm mitochondria are particularly vulnerable, leading to reduced motility. Additionally, Leydig cell ATP levels decrease, impacting testosterone synthesis and secretion and increased lactate dehydrogenase release. These effects may impair reproductive function in long-term Li + treatment. Dysfunction in sperm mitochondria may result from disrupted membrane potential and electron transport chain interference (Filippa and Mohamed, 2019; Yousefsani et al., 2020), as well as increasing mitochondrial permeability and facilitating the release of various cell death mediators into the cytoplasm. Recently, safe and clinically applicable agents like amino acids and peptides enhance mitochondrial function and energy metabolism. (Ben Saad et al., 2017; Jamshidzadeh et al., 2017; Heidari et al., 2019). Further studies are warranted to elucidate the precise mechanisms underlying Li^+-

induced reproductive organ injury, potential interactions of adjunctive treatments with Li^{+}'s pharmacological effects, and the translation of experimental findings into clinical practice.

4.2 Antipsychotics (Aps)

Individuals with psychosis often require lifelong treatment with AP, which can lead to various side effects, including abnormal movements, weight gain, diabetes, and reproductive disorders like menstrual irregularities or amenorrhea in women (Elmorsy et al., 2017a). Despite past beliefs that typical APs were more toxic than atypical ones, research suggests that both types show similar reproductive toxicity (Murke et al., 2011).

Notably, APs can lead to reproductive disorders through hyperprolactinemia, primarily induced by dopamine D2 receptor inhibition. While typical antipsychotics are often associated with hyperprolactinemia (about 57% of patients), atypical ones generally do not affect prolactin levels except for risperidone (Wong, 2007). However, there's no direct correlation between prolactin levels and menstrual irregularities (Lee and Kim, 2006). Regardless of hyperprolactinemia status, antipsychotics are linked to similar rates of reproductive dysfunction and may reduce peri-ovulatory estradiol levels (Canuso et al., 2002). Hence, while hyperprolactinemia is significant, it may not fully explain antipsychotic-induced reproductive toxicity.

APs induce cytotoxic effects in rat ovarian theca interstitial cells by inhibiting mitochondrial bioenergetics. Both *in vitro* (Elmorsy et al., 2014) and *in vivo* (Martins et al., 2008) studies demonstrate that APs induce oxidative stress in non-reproductive cells (Elmorsy et al., 2017a; Elmorsy et al., 2017b).

The study explores how antipsychotics (APs) induce reproductive toxicity via oxidative stress in rat ovarian theca interstitial cells (TICs). APs cause cell damage, increased caspase activity, and oxidative stress (high ROS production, reduced glutathione levels, and heightened lipid peroxidation). Antioxidants alleviate this damage, suggesting a potential therapeutic approach, but clinical research is needed for future validation (Elmorsy et al., 2017a).

Schizophrenia, a worldwide condition marked by symptoms like hallucinations and confusion, often starts during the reproductive years, affecting brain neuroendocrine functions and disrupting reproductive processes (Ardıç et al., 2021). Antipsychotic medications used for schizophrenia treatment can cause reproductive toxicity by affecting hormonal regulation, leading to sexual dysfunction, disrupted spermatogenesis, and abnormalities in epididymal maturation (Solomon et al., 2019; Zhao et al., 2019). Olanzapine (OLZ), a second-generation antipsychotic used for schizophrenia and bipolar disorder treatment, affects various neurotransmitter systems. It acts on multiple neurotransmitter receptors, including dopamine, serotonin, muscarinic, adrenergic, and histaminergic receptors. It can increase prolactin levels in females, leading to adverse effects like amenorrhea, impotence, and sexual dysfunction (Fernandes et al., 2019; Meftah et al., 2020). Elevated prolactin levels can cause hypogonadism hindering sperm production and causing issues like delayed spermatogenesis, reduced semen quality, and abnormal testicular tissue in both humans and animals (Akram et al., 2019; Zhao et al., 2019). Research on male rats showed that OLZ administration reduced normal sperm morphology and caused toxicity in testicular tissue, attributed to increased oxidative stress, Leydig cell damage, and disruption of hormone regulation (Ardıç et al., 2021). Of particular importance, Elevated ROS levels in 25% of infertile males contribute to sperm defects and dysfunction, affecting sperm functions like capacitation, acrosome reaction, mitochondrial sheath stability, and motility (Sikka and Hellstrom, 2017). Sperm cells' susceptibility to ROS is attributed to their high levels of unsaturated fatty acids in the membrane and limited cytoplasmic ROS-neutralizing enzymes (Sidorkiewicz et al., 2017). Lipid oxidation can lead to compromised cell membrane integrity, heightened membrane permeability, enzyme inactivation, DNA impairment, and ultimately, cell apoptosis (Sikka and Hellstrom, 2017), potentially leading to decreased sperm count, activity, motility, and abnormal sperm morphology (Sidorkiewicz et al., 2017). Following olanzapine treatment, testicular GSH levels decreased notably in addition to a significant increase in SOD activity in the olanzapine-treated group (Ardıç et al., 2021). Subsequently, free radicals induce sperm oxidative stress, impairing function and fertility. Elevated ROS levels in the testes lead to semen oxidative stress, associated with idiopathic infertility. OLZ reduces GSH levels, indicating oxidative stress, while increased SOD levels suggest a rapid ROS response. High doses of OLZ exacerbate oxidative stress, impacting sperm morphology and testicular structure (Simon and Carrell, 2013; Elghaffar et al., 2016). More research needed to understand OLZ's reproductive toxicity. Monitoring sperm and hormone levels in patients is essential for risk assessment (Ardıç et al., 2021).

4.3 Antiepileptics

Epilepsy, affecting 20–40 million people worldwide, is characterized by abnormal neuronal activity and is managed with long-term medication using antiepileptic drugs (AEDs), tailored to individual needs (Asif, 2017). While newer AEDs offer improved tolerability, challenges such as adverse effects and drug interactions persist (Fisher et al., 2005). Certain AEDs may impact reproductive health, contributing to reproductive disorders (Isojärvi et al., 2004). The pharmacological landscape of AEDs has expanded with advancements in drug design and insights into seizure mechanisms (Mohanraj and Brodie, 2003). Epilepsy management requires a comprehensive approach that addresses therapeutic gaps and minimizes adverse effects (Kwan and Sander, 2004). Reproductive dysfunction, including reduced fertility in both sexes, is prevalent among epileptic patients, possibly due to epilepsy itself or antiepileptic medication (Artama et al., 2004). Catamenial epilepsy, influenced by hormonal changes, exhibits estrogen-induced seizures and progesterone's anticonvulsant effects (Isojärvi et al., 2004). Thyroid hormones also affect seizure activity, with thyrotoxicosis possibly increasing seizure risk (Asif, 2017). Epilepsy correlates with various reproductive disorders, including irregular menstrual cycles and decreased potency which are exacerbated by untreated epilepsy (Asif, 2017). Seizures and interictal periods disrupt hormone release, affecting reproductive function. AEDs may also impact reproductive hormones, causing menstrual disorders, reduced potency, and

diminished sexual interest (Asif, 2017). Limited data exist on newer AEDs like Oxcarbazepine and their reproductive effects.

Women with idiopathic generalized epilepsy (IGE) have higher rates of reproductive disorders like polycystic ovaries (PCO), hirsutism (HA), and polycystic ovary syndrome (PCOS) compared to those with localization-related epilepsy (LRE) or without epilepsy (Morrell et al., 2002). IGE is linked to anovulatory cycles, polycystic appearing ovaries, elevated BMI, and HA. In contrast, LRE is associated with PCOS related to left-sided focus and hypothalamic amenorrhea and hyposexuality linked to right-sided focus (Asif, 2017). Epilepsy disrupts pituitary hormone regulation, with left-sided focus epilepsy linked to disturbances in the temporo-limbic hypothalamic-pituitary axis, with left-sided focus epilepsy showing increased LH secretion and LH/FSH ratio (Herzog et al., 2003). Prenatal genetic factors may influence epilepsy and hormone regulation, potentially creating a bidirectional relationship with reproductive disorders (Asif, 2017).

4.3.1 Valproic acid

In women with epilepsy (WWE), valproic acid (VPA) use predicts reproductive disorders like polycystic ovary syndrome (PCOS), hirsutism (HA), and polycystic ovaries (PCO). Starting VPA at a younger age correlates with increased HA and PCOS incidence, while obesity does not significantly predict reproductive issues (Asif, 2017). Regression analysis helped isolate these factors' effects, consistent with previous research reports (Morrell et al., 2002; Isojärvi et al., 2004). Further research is needed to confirm and explore factors influencing reproductive health in WWE, as few studies have utilized regression analysis to identify contributing factors (Morrell et al., 2002; Herzog et al., 2003; Mikkonen et al., 2004).

WWE had comparable fertility rates to controls, but lower fertility if epilepsy persisted into adulthood. MWE also experienced reduced fertility with active epilepsy in adulthood. Few population-based studies have explored epilepsy's impact on fertility despite its correlation with reproductive disorders (Artama et al., 2004). Active epilepsy and medication during adulthood were associated with reduced fertility in WWE, while remission before adulthood led to similar fertility rates as controls (Mikkonen et al., 2004).

Furthermore, Girish et al. (2014) examined sodium valproate-induced reproductive toxicity in male rats (Girish et al., 2014). Treatment at 400 mg/kg/day led to reduced body and testis weights, decreased sperm count and motility, and histological changes in the testes, including necrosis, atrophy in seminiferous tubules, and impaired spermatogenesis, as well as a notable reduction in Johnsen's testicular score (Bairy et al., 2010; Cansu, 2010; Girish et al., 2014). Notably, sodium valproate disrupts cellular mechanisms, potentially inducing free radical formation and lipid peroxidation, which may disrupt testicular structure and function (Tamber and Mountz, 2012). Additionally, it promotes apoptosis in human and rat granulosa cells by increasing caspase-3 activity (Cansu, 2010).

Turning to developmental toxicity caused by VPA, it induces extensive transcriptional changes, with downregulated genes associated with RNA processing and chromatin modification/histone acetylation, consistent with its known histone deacetylase (HDAC) inhibitory action. It is classified as a developmental toxicology chemical, notably down-regulating mRNA expression of neuronal markers like NF-68, NF-200, NMDA-receptor, and GABAA-receptor. Moreover, therapeutic plasma concentrations in adults and children upregulate nestin mRNA expression, suggesting glial cell activation or neural precursor cell proliferation in response to neuronal cell death (Krug et al., 2013).

4.3.2 Pregabalin

Pregabalin, six times more potent than Gabapentin, is widely used in psychiatry and neurology for treating epilepsy, anxiety, fibromyalgia, and neuropathic pain in diabetic patients. Acting on voltage-gated calcium channels, it provides analgesic and anxiolytic effects, also influencing the dopaminergic reward system. Despite misuse potential, its prescribed use is escalating (Shokry et al., 2020). Yet, when given at therapeutic doses to non-abusers, its abuse potential may be lower compared to benzodiazepines, stimulants, or opioids (Loftus and Wright, 2014). Pregabalin does not enhance euphoria or sexual ability; it may cause dysfunction, especially in epilepsy patients. Tramadol is highly toxic, affecting seminiferous tubules and sperm severely. Morphine, hashish, and heroin also impair sperm quality, with morphine's effect on count and motility debated (Isojärvi et al., 2005). For instance, a study found that pregabalin negatively impacted male rat reproductive function, reducing serum testosterone levels without affecting pituitary gonadotropins. Histopathological examination revealed significant degenerative changes in the seminiferous epithelium and decreased cell counts for all spermatogenesis cells, Sertoli cells, and Leydig cells. Immunohistochemical analysis indicated increased apoptosis, shown by caspase3 expression (Shokry et al., 2020). Interestingly, Pregabalin toxicity may involve central action, inhibiting the hypothalamic-pituitary-gonadal axis. It decreases melatonin levels, reducing Leydig cell protection against oxidative stress and diminishing testosterone release. Additionally, its impact on serotonin leads to reduced thyroid hormone levels, further impairing Leydig cell development. Oxidative stress, implicated in apoptosis induction, plays a significant role in pregabalin toxicity (Zhao and Wang, 2012).

In female rats, pregabalin reduced pituitary gonadotropins but increased E2, progesterone, and testosterone levels. Ovarian histopathology showed increased atretic follicles and heightened apoptosis, consistent with barbiturates inducing ovarian atrophy. Tawfeeq et al. observed dose-dependent LH, FSH, and prolactin inhibition with pregabalin inhibition, correlating with elevated atretic ovarian follicles (Tawfeeq et al., 2016). Pregabalin's toxicity affects the pituitary gland, stimulating E2 secretion or inhibiting progesterone's effect on hypothalamic GnRH. Other drugs, like tramadol, induce ovarian failure, while oxymorphone partially inhibits ovulation (Shuey et al., 2008; El-Ghawet, 2015; Shokry et al., 2020).

4.3.3 Anticonvulsant drugs

Anticonvulsant drugs like phenobarbital (PBT), phenytoin (PHT), and carbamazepine (CBZ) are associated with teratogenic effects when used during pregnancy, leading to major malformations, microcephaly, growth retardation, and minor facial and finger abnormalities in infants (Isojarvi, 2001; Perucca, 2004). The risk of congenital malformations is doubled in children born to mothers taking AEDs, with specific syndromes like fetal

hydantoin syndrome (associated with PHT) and spina bifida (linked to VPA) highlighting these risks. Balancing the need to minimize AED exposure against controlling maternal seizures is crucial during pregnancy, with close monitoring recommended for pregnant women on AEDs (Wagh et al., 2011; Asif, 2017).

While first-generation AEDs like VPA and CBZ are known for teratogenic effects, limited data exist for newer compounds like ethosuximide (ETH) and levetiracetam (LEV)) (Pennell et al., 2012). ETH has shown teratogenicity and cognitive impairment in rodents, while LEV has demonstrated developmental effects only at very high doses (Mawhinney et al., 2013). Recent findings suggest LEV may be a safer alternative to VPA, with a lower risk of major congenital malformations in women with epilepsy of childbearing age.

A previous report investigated Zebrafish as a valuable model for assessing developmental toxicity, offering advantages for medium/high-throughput screening as an embryonic/larval model. A study aimed to enhance the predictability of the zebrafish model by employing an integrated screening strategy with various endpoints, including morphology, behavior, histopathology, kinetics, and phenotyping through *in situ* hybridization. Four AEDs (VPA, CBZ, ETH, and LEV) were selected as model compounds (Beker van Woudenberg et al., 2014). Histopathological analysis of VPA-treated larvae revealed reduced brain cellularity, particularly in the optic regions, highlighting histopathology as a sensitive endpoint. Motor activity analysis confirmed VPA's neurodevelopmental toxicity, supported by literature evidence. CBZ primarily affected hatching and motor activity at lower concentrations, aligning with rodent and human studies indicating CBZ-induced neurodevelopmental disorders. ETH-induced pericardial edema and neurodegeneration, with biphasic dose-response effects observed. LEV exhibited significant motor activity effects at lower concentrations, suggesting its developmental neurotoxic potency, despite minimal structural damage (Kultima et al., 2010; Beker van Woudenberg et al., 2014).

Additionally, expression patterns of Aldh1a2 and Cyp26a1 indicated a link between gene expression and apical endpoints, suggesting the potential use of molecular markers for phenotype prediction (Menegola et al., 2006; Beker van Woudenberg et al., 2014).

Recent studies suggest LEV may harm sperm quality in rats, causing reduced concentration, motility, abnormal morphology, DNA damage, and testicular tissue damage in male rats via oxidative stress and hormonal imbalances. Human studies are needed to assess LEV's reproductive risks and fertility impact. Clinical research should focus on reproductive toxicity and fertility in LEV-treated individuals, evaluating sperm DNA damage and oxidative status (Baysal et al., 2017).

4.4 Cholinergic toxicity

Various factors like toxic agents, malnutrition, illness, or stress can disrupt male reproductive function, impairing fertility via androgen production, germ cell development, and somatic cell maintenance. Cholinergic signaling proteins, vital for sperm function, are expressed in male reproductive tissues. Dysfunction, possibly induced by agricultural agents or stress, can harm fertility. Chemotherapeutic drugs may also affect cholinergic proteins, underscoring the need to understand cholinergic toxicity in male fertility (Mor and Soreq, 2011).

Spermatogenesis in testicular seminiferous tubules involves Leydig cells producing testosterone and Sertoli cells maintaining the microenvironment. It progresses through stages from spermatogonia to spermatozoa. Cholinergic innervation aids sperm transport and regulates production via acetylcholine, acting on receptors in smooth muscles. Nicotinic receptors are in parasympathetic ganglia, and muscarinic receptors are in smooth muscles, with acetylcholinesterase terminating signaling (Mor and Soreq, 2011).

Nicotinic acetylcholine receptors (nAChRs) in sperm aid fertilization and regulate motility, influenced by testicular proteins. nAChRs are involved in regulating Leydig cell function and testosterone secretion (Kumar and Meizel, 2005). Muscarinic acetylcholine receptors (mAChRs) in epithelial cells of sperm and Sertoli cells affect proliferation and luminal fluid composition (Avellar et al., 2010).

AChE has diverse isoforms like AChE-R and N-AChE, differing in their C- and N-domains due to alternative splicing and promoter usage. AChE-R is found in human and mouse sperm and is linked with differentiation by containing pseudointron I4, interacts with cellular proteins, and correlates with sperm motility. N-AChE, found in round spermatids' acrosome, is involved in spermatogenic differentiation (Mor and Soreq, 2011). Exposure to anti-cholinesterase pesticides like malathion, dimethoate, and chlorpyrifos has been linked to testicular toxicity in rodents, affecting sperm counts, testosterone levels, and testicular histology, possibly through disruption of cholinergic signaling in Leydig cell function (Joshi et al., 2007; Choudhary et al., 2008; Ruchna Verma and Banalata Mohanty, 2009).

4.5 Molindone-induced reproductive toxicity

Molindone hydrochloride, a dopamine D2 and serotonin 5-HT2B receptor antagonist, is under investigation for treating impulsive aggression (IA). Developed for schizophrenia, it is now being studied as SPN-810, an extended-release version, for IA in attention-deficit/hyperactivity disorder. Rat studies reported CNS signs and prolactin increases. While developmental toxicity is not evident, its effects on fertility are unclear. Dopamine antagonism-induced prolactin elevation may affect reproduction differently among species, necessitating further evaluation, particularly in rats, for accurate human risk assessment (Krishna et al., 2017).

Regulatory-compliant DART studies on molindone HCl, a dopamine D2 receptor antagonist, found no teratogenicity or adverse fetal effects in rats (up to 40 mg/kg/day) and rabbits (up to 15 mg/kg/day) during organogenesis. These doses, with exposure margins of 69X and 6X over clinical levels, were well-tolerated (Gopalakrishnan et al., 2018).

Furthermore, a postnatal development study revealed no effects on survival, developmental milestones, or functional evaluations in rats. Transient reductions in pup weight gain were observed at the highest dose, but no post-weaning effects or impact on litter size. Maternal hypoactivity is possibly linked to a slight reduction in pup survival. Molindone HCl induced CNS-related signs and maternal

toxicity in rats and rabbits, consistent with its pharmacology. Fertility studies in rats showed altered estrous cycle duration but no effects on mating or reproductive parameters in males or females (Gopalakrishnan et al., 2018). These effects, attributed to dopamine D2 receptor antagonism and prolactin secretion, are not considered relevant to human reproduction. Clinical trials support the reproductive safety of molindone HCl in target patients (Stocks et al., 2012; Gopalakrishnan et al., 2018).

4.6 Nanoparticles (NPs) and drug delivery nanocarriers

Nanotechnology in nervous system imaging and drug delivery offers precise treatment but raises concerns about nanoparticles' (NPs) health risks (Pinheiro et al., 2021; Faiz et al., 2022; Jo et al., 2022; Qiao et al., 2023). NPs from various products may accumulate in tissues, posing risks to pulmonary, liver, kidney, and neurological issues. Their ability to breach barriers like the blood-brain barrier and the placenta raises concerns about reproductive health and fetal development (Hersh et al., 2022). In reproductive medicine, Gold nanoparticles aid cell visualization in ovarian carcinoma, and tocotrienol nanosized emulsions treat breast and ovarian tumors. NP exposure can affect sperm count, morphology, hormonal levels, and sexual behavior in males, and ovarian function in females (Ahmad, 2022). Public awareness about NP toxicity is crucial for both genders' reproductive health and fetal development. NPs can disrupt the female reproductive system, governed by hormones, potentially leading to fetal abnormalities. Studies indicate acute and chronic toxic effects on reproductive tissues, highlighting concerns (Souza et al., 2021). NPs can penetrate biological barriers like the placenta, affecting male and female reproductive tissues. Zebrafish models offer insights into embryonic development due to ethical constraints with traditional animal models (Blum et al., 2012). NPs can disrupt reproductive function, leading to developmental and fertility issues. Research shows adverse effects on maternal weight, placental health, implantation rates, and hormone levels, impacting pregnancy outcomes (Brohi et al., 2017). In pregnant mice, cadmium oxide NPs delayed maternal weight gain and impacted placental weight, possibly affecting implantation. Silica and titanium oxide NPs reduced uterine weight and increased fetal reabsorption rates, suggesting adverse effects on reproductive tissues and fertility (Hou and Zhu, 2017).

4.6.1 Effects of gold and titanium dioxide NPs on the male reproductive system

Male reproductive health is affected by NPs at molecular, cellular, and histological levels, requiring thorough toxicity evaluations (Habas et al., 2021). Titanium dioxide NPs during mouse pregnancy altered male offspring's neurological tissues and affected Leydig and Sertoli cells, impacting reproductive growth (Umezawa et al., 2012). Intra-tracheal carbon-based NP exposure during pregnancy induces histopathological changes in seminiferous tubules, affecting sperm production in male offspring. Multi-walled carbon nanotube exposure causes reversible testicular damage without affecting fertility in mice (Iftikhar et al., 2021). Testicular nanoparticle accumulation impacts germ cell numbers, histopathology, and sperm motility. Water-soluble NPs have fewer toxic effects, while fat-soluble ones can induce apoptosis or inflammation, compromising male fertility (Hong et al., 2016).

Gold NPs in semen impaired sperm motility, while polyvinyl alcohol-coated iron oxide nanoparticles had no effect (Vassal et al., 2021). *In vitro* models using mouse and bovine sperm showed nanoparticle cytotoxicity, with silver nanoparticles most toxic (Mohammadinejad et al., 2019). Airborne nanoparticulate pollutants from industry pose reproductive health risks, evident in germ-line mutational changes in mice exposed to such pollutants (Jaishankar et al., 2014).

4.6.2 Effects of lead and zinc NPs on the male reproductive system

Lead, a highly toxic heavy metal, is widespread in the environment due to human activities and poses significant health risks with neurotoxic and immunotoxic effects (Mahmood et al., 2012). Zinc oxide nanoparticles, valued for their biocompatibility, are used in products like sunscreens but can penetrate cells, exhibiting toxicity influenced by size and dosage (Keerthana and Kumar, 2020; Deore et al., 2021).

In research, the detrimental impacts of lead and zinc oxide nanoparticles on experimental animals' reproductive organs have been elucidated, with outcomes dependent on dosage and exposure duration (Lee et al., 2016). Exposure to zinc oxide nanoparticles has been associated with notable declines in sperm counts and motility, likely due to induced oxidative stress (Lee et al., 2016). Earlier research has also highlighted toxic effects on the testis and epididymis from both zinc oxide nanoparticles and lead. (Deore et al., 2021). Indeed, oxidative stress emerges as a pivotal factor in metal-induced toxicity, marked by an imbalance between free radicals and antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase (Ighodaro and Akinloye, 2018). This imbalance can precipitate organ toxicity and is implicated in various health conditions. Increased oxidative stress has been observed in the male reproductive systems following exposure to lead and zinc oxide NPs, underscoring their involvement in the toxic effects of these metals (Ighodaro and Akinloye, 2018; Deore et al., 2021).

4.6.3 Effects of NPs on the female reproductive system

NPs' toxic effects include female reproductive disruption, teratogenicity, and prenatal development issues, as they translocate to reproductive and fetal tissues through inhalation, ingestion, or dermal absorption (Dugershaw et al., 2020). Titanium dioxide (TiO_2) NPs disrupted granulosa cell hormonal secretions, reducing pregnancy rates and altering ovarian gene expression in mice (Chen et al., 2003). Exposure to zinc oxide NPs during pregnancy or lactation poses health risks to mothers and embryos (Clementino et al., 2021). Nanoparticles accumulate in ovarian tissues in a size-dependent manner, with larger particles accumulating more than smaller ones (Raj et al., 2017). NPs affect oogenesis based on factors like size, surface charges, and exposure routes. Metals, metallic oxides, carbon-based NPs, and quantum dots penetrate female germline cells, inducing reactive oxygen species, DNA damage, and inflammation. Gold and silver NPs, including their alloys, accumulate in oocytes and cumulus cells, with silver NPs showing higher toxicity (Mao et al., 2022).

NPs have adverse effects on the female hormonal system by disrupting the hypothalamus-pituitary-ovarian axis, leading to

neuro-hormonal instabilities (Hou and Zhu, 2017). Nickel NPs in rats disrupt hormones and damage ovaries, while titanium dioxide NPs affect hormone levels and follicles in female rats (Karimipour et al., 2018). Furthermore, quantum dots and calcium phosphate NPs disrupt ovarian cell activities and steroid synthesis pathways. NPs crossing cellular barriers impact placental function and fetal development, influenced by barrier thickness. Evolving placental barriers regulate substance exchange between maternal and fetal compartments (Woods et al., 2018). Understanding NP-biological barrier interactions is crucial for safer nanoparticle therapies (Herrick and Bordoni, 2019). Drug-delivering NPs can induce developmental toxicities, affecting fetal cell growth, differentiation, and genetic expression. Crossing placental barriers causes neurodevelopmental anomalies and DNA damage. *In utero* gene editing with multifunctional nanoparticles offers therapeutic promise with no fetal developmental adverse effects (Ricciardi et al., 2018). However, NPs may cause fetal abnormalities, with variations in toxicity among different types. Some, like amorphous silica-based NPs, exhibit no prenatal toxicities, while others, such as molybdenum-based NPs, impact maternal weight, fetal growth, and genetic stability (Mohamed et al., 2020).

Regulatory frameworks for nanoparticle reproductive toxicity need integration. Harmonized guidelines are essential for assessing risks to reproductive health from nanoparticles, requiring comprehensive data for clinical safety assessment (Grillo et al., 2021). Reproductive toxicity studies may be needed in later clinical trial phases based on product and patient considerations (Husain et al., 2015).

4.7 Opioids

Opium use, prevalent during events like the SARS-CoV-2 pandemic, poses risks to cognition and reproductive health (Khosravi, 2022). Morphine, its main alkaloid, disrupts hormonal balance via testicular opioid receptors, impairing sperm production and quality. Morphine induces ROS, damaging cell membranes and causing DNA fragmentation. Long-term use leads to dependence by affecting brain receptors, affecting key structures like the amygdala and hippocampus, and testicular opioid receptors, disrupting hormonal balance (Moinaddini et al., 2023). Replacement therapies like methadone and buprenorphine manage dependence. Methadone aids detox but can cause side effects, while buprenorphine, favored since 2001, has minimal placental transfer in pregnant women (Meyer et al., 2015; Robin et al., 2022; Moinaddini et al., 2023).

A study investigated sperm and testis parameters in morphine-dependent animals and those undergoing detoxification with methadone/buprenorphine (Moinaddini et al., 2023). Morphine affects testicular opioid receptors, impacting germ and glandular cells, increasing DNA fragmentation, and potentially causing infertility. Morphine use and detox affect mitochondrial activity and sperm motility; while improved viability is seen with methadone detox. Testicular weight and dimensions decrease with morphine use and detox (Moinaddini et al., 2023).

Chronic opium use in male rats alters testicular architecture, inducing inflammation and toxicity. Opium addiction reduces sexual activity via decreased testosterone levels, potentially causing sexual suppression and infertility. Hypophysial gonadal secretion function decreases, impacting sperm quality, which may result from direct action on gonads or via the hypothalamic-hypophysial-gonadal axis, affecting proper spermatogenesis and male sexual responses. Suppression of this axis results in reduced sperm count, semen quality, erectile function, and infertility. Further research is needed to understand opium's reproductive health effects (Hejazian et al., 2007; Amin, 2013).

5 Examining neurotherapeutic drug-induced reprotoxicity via organoid modeling: Steps toward personalized therapy

There is a significant gap in the literature regarding the assessment of neurotherapeutic drug-induced reprotoxicity using organoid modeling. This limitation underscores the crucial need to develop and use reproductive organoids to study the toxic effects of neurotherapeutic drugs on reproduction. Moreover, it highlights the importance of incorporating organoid modeling into research methodologies rather than conventional approaches based on cell lines and mice to develop more clinically relevant and predictive models.

Antidepressants may affect semen parameters and male fertility, with mirtazapine potentially exerting fewer adverse effects on germ cell DNA damage than amitriptyline. However, studies based on germ cell lines may have limited human relevance. Further research on the effects of antidepressants on semen quality and fertility is crucial. Testicular organoids offer a promising solution for reducing animal use in toxicity studies, thereby addressing limitations in current clinical indicators for testicular toxicity. These 3D structures mimic the intricate cell interactions in the testes, providing a valuable tool for modeling normal development and pathophysiology and performing drug testing. Thus, testicular organoids hold immense potential for assessing reproductive toxicity while minimizing animal use and associated costs.

Wu et al. (2022) established an enhanced testicular organoid model comprising rat testicular cell homogenates to evaluate the reproductive toxicity of antidepressants, focusing on mimicking drug effects on various aspects of spermatogenesis and elucidating underlying mechanisms. Using such a model, amitriptyline and mirtazapine were selected, the two most commonly used antidepressants, to assess their impact on spermatogenic cells (Wu et al., 2022).

Testicular organoids were used to examine the effects of antidepressants. Compared with mirtazapine, amitriptyline induced greater apoptosis in the organoids with higher cell death rates. Amitriptyline exhibited greater cytotoxicity across cell lines than mirtazapine, indicating its higher potential for testicular damage (Wu et al., 2022).

Immunostaining confirmed the presence of germ cells and Sertoli cells, suggesting spermatogenesis initiation and BTB formation (Yuan et al., 2020). In the abovementioned study, both amitriptyline and mirtazapine exerted dose-dependent effects on spermatogenesis-related gene expression, with amitriptyline inducing greater toxicitythan mirtazapine (Yuan et al., 2020). Extended mirtazapine treatment suggested potential damage to

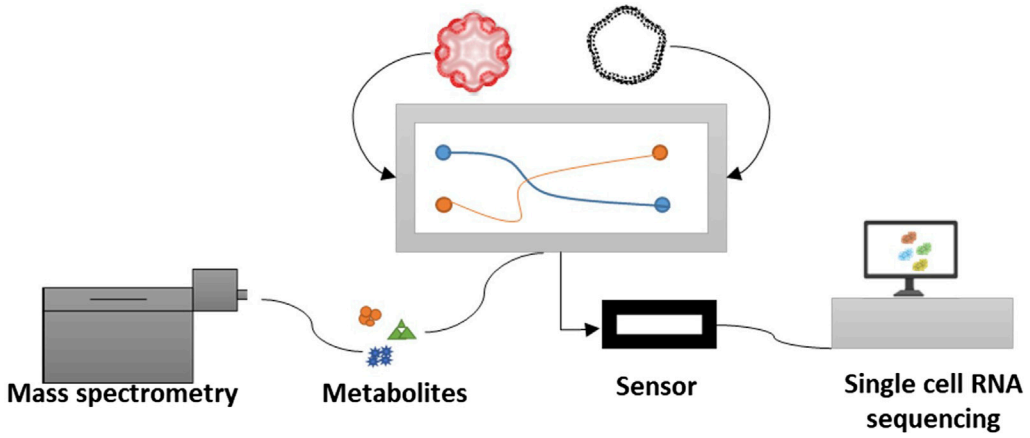

FIGURE 3
Integrated Multi-Organoid System on Chip: This model combines micro-engineered organoids (e.g., brain and reproductive organs) on chips with single-cell RNA sequencing (sc-RNA-seq) and mass spectrometry to investigate the systemic toxicity of neurotherapeutic drugs. Therapeutic compounds are introduced into the model to target brain diseases and assess their effects on reproductive organoids.

undifferentiated spermatogonia (Yuan et al., 2020). At lower doses, none of the drugs affected Zo1 expression, preserving BTB integrity, whereas amitriptyline downregulated Zo1 expression at higher doses, indicating BTB damage (Yuan et al., 2020). Sertoli cells displayed resistance to drug-induced damage, particularly for mirtazapine (Wu et al., 2022). These findings underscore differing reproductive toxicity profiles of amitriptyline and mirtazapine, highlighting the need to assess drug effects on testicular function and spermatogenesis.

The organoid platform evaluates antidepressant toxicity on spermatogonia, with amitriptyline showing more significant effects on spermatogenesis genes than mirtazapine. Comparisons with mouse cell lines support these findings, validating the organoid model's relevance. Despite refinement needs, the model is a valuable tool for drug toxicity screening and mechanistic studies on spermatogenesis, offering high repeatability and ease of operation. Pioneering research on rat testicular organoids may be translated to human models, elucidating clinically relevant drug reproductive toxicity (Wu et al., 2022).

Furthermore, lamotrigine, a commonly used drug for treating various neurological conditions, such as epilepsy and bipolar disorder, is associated with potential side effects, including adverse effects on reproductive health such as disrupted menstrual cycles, hormonal imbalances, and alterations in fertility parameters. Furthermore, emerging evidence suggests that lamotrigine can exert toxic effects on reproductive organs, particularly the endometrium (Ann et al., 2023; Mwangi et al., 2023; Rezk et al., 2024).

6 Advancements and challenges in assessing developmental toxicity

Human organoids, although promising, encounter challenges such as inconsistent batch outcomes and incomplete maturity, affecting their reproducibility and ability to replicate native organ functionality. Addressing the limitations of batch variability and heterogeneity through standardized protocols could enhance the reliability and applicability of human organoids (Velasco, Kedaigle et al., 2019). Additionally, efforts to augment organoid complexity involve integrating vascular and immune components to better mimic native tissue structures and functions (Hofer and Lutolf, 2021). Engineering techniques create vascularized and immune organoids, like brain organoids with microglia-like cells (Popova et al., 2021). Additionally, Biomaterials and microfluidic systems mimic *in vivo* cellular environments within organoids (Li et al., 2022).

Another notable advancement is the emergence of "organs-on-chips" (OoCs) platforms, which faithfully replicate the dynamic microenvironment of human organs, facilitating *in vitro* assessment of systemic toxicity (Figure 3). OoC devices replicate tissue functions, aiding in understanding physiological dynamics and multi-organ connectivity. Mathematical modeling quantifies responses, while lab-on-a-chip platforms integrate microfluidic chips for dynamic cultures. In biosciences, *in silico* and theoretical modeling refine systems, with MoC systems modeling toxin processes for systemic toxicity insights. (Devall et al., 2021; Sung, 2022). For instance, embedding JEG3 on a chip can interact precisely with HUVECs, resembling the placenta unit in maternal-fetal interface studies (Mittal et al., 2019; Cui et al., 2020). Moreover, high-throughput single-cell RNA sequencing (scRNA-seq) has been instrumental in characterizing the transcriptome of individual cells within organoids, allowing researchers to identify alterations induced by toxic exposures. For example, scRNA-seq has been used to study the effects of prenatal exposure to toxic substances on cell development and differentiation (Wang et al., 2021). Despite its promise, the integration of scRNA-seq into developmental toxicity assessment with organoids remains limited (Devall et al., 2021).

Ferasyi et al. demonstrated the complexity of reproductive signaling pathways with a male reproductive axis model, highlighting hormone interactions among the central nervous system, pituitary gland, and gonads. Mathematical modeling elucidates fluid dynamics, with lumped-parameter models

resembling electrical circuits and hydrodynamic models analyzing biofluid flows directly using the Navier-Stokes equation (Mazumdar, 2015; Ferasyi et al., 2016). Furthermore, pharmacokinetic (PK) and pharmacodynamic (PD) models predict compound effects on the endocrine system. PB PK-PD models, using ODEs, integrate signaling processes with mathematical relationships like Hill and Michaelis-Menten equations. Systems biology-based algorithms simulate network models, aiding drug screening in OoCs (Schnell, 2014).

Turning to microfluidics, it Microfluidics aids MEMS device design for biomedicine, handling nanoliter volumes and integrating sensors (Karolak et al., 2018; Manz et al., 2020). Laminar flow, influenced by shear stress, governs fluid behavior, with diffusion ensuring stable gradients. Computational fluid dynamics predict behavior, while tissue-mimicking chips study molecular events (Morshed and Dutta, 2018; Piemonte et al., 2018; Manz et al., 2020; Sung, 2022). Interestingly, multi-organ systems mimic interactions via microfluidic connections, aided by PBPK-PBPD models. Scaling optimizes design for predicting responses (Lee et al., 2017; Prantil-Baun et al., 2018).

Turning our attention to the reproductive tract, it is complex, housing gonads and vital organs like the ovary. Microfluidic biochips mimic ovulation, providing insights into infertility pathways. OoC devices integrate endocrine loops, aiding the study of endocrine-disrupting chemicals (EDCs)-triggered pathways with *in silico* methods (Xiao et al., 2017; Bodke and Burdette, 2021).

A novel endometrium-on-a-chip device simulated cyclic estradiol hormone effects on stromal and endothelial cells. It featured dual-chamber microfluidics with a porous membrane for co-culturing, maintaining steroid sensitivity for biochemical analysis. Endocrine organ-on-chip systems by Nguyen et al. and Gnecco et al. could benefit from integration with *in silico* algorithms or mathematical models, as shown by Lee et al. in a pancreas-muscle-liver OoC (Gnecco et al., 2017; Lee et al., 2017; Nguyen et al., 2017).

OoCs replicate dynamic hormone signaling in microfluidic environments, mimicking human reproductive pathways (Nawroth et al., 2018). Xiao et al. (Xiao et al., 2017) conducted a study combining microfluidic culture of the human reproductive tract with mathematical PK simulation. Their system orchestrated synchronized fluid flows to emulate the menstrual cycle hormone profile. Ovarian follicles generate hormones regulating downstream tissues, with an ODE system modeling inter-organ hormonal signaling for drug discovery and toxicological studies (Sung, 2022).

Additionally, the integration of omics technologies enhances the understanding of developmental toxicity mechanisms and facilitates biomarker discovery (Mao et al., 2024). Integration of mass spectrometry with omics technologies and organoid models enhances assay precision, refining compound potency ranking in reprotoxicity tests. Mass spectrometry provides insights into developmental toxicity mechanisms, identifying biomolecules, metabolic pathways, and biomarker signatures in organoids exposed to toxic compounds. This integration improves sensitivity and specificity, aiding in early detection and prediction of adverse developmental outcomes (Abady et al., 2023; Xu and Yang, 2024). This synergistic approach advances our ability to evaluate and mitigate developmental hazards in pharmaceutical and environmental contexts.

More research is needed to enhance reproductive disease modeling. The epididymis is pivotal for sperm maturation, but our understanding is limited. Few 3D epididymal organoids exist, with challenges including the blood-epididymis barrier's integrity for drug testing. Additionally, mimicking the different portions of the epididymis remains a significant challenge, highlighting the need for further research in this area (Leir et al., 2020; Pinel and Cyr, 2021; Patrício et al., 2023).

Emerging research focuses on embryoids, organized embryo-like structures aiming to model integrated early embryonic development. Unlike organoids, they offer reproducible cellular organization and architecture within a shorter timeframe (Fu et al., 2021). Gastruloids, for example, serve as models of gastrulating embryos, providing insights into anterior-posterior axial patterning and potential teratogenicity assessment (Papalia et al., 2007; Mantziou et al., 2021). Saadeldin et al. (2024) conducted an experiment involving the co-cultivation of endometrial organoids (EOs) with embryos and made several significant observations. Notably, it was observed a five-fold increase in the cell number of co-cultured embryos and a seven-fold increase in the proportion of trophoblast outgrowths compared to control embryos. Additionally, embryos cultured with an EO-conditioned medium demonstrated a higher rate of attachment compared to other models, and remarkably, embryonic elongation was observed for the first time, providing a valuable tool for investigating the intricate processes involved in porcine embryo implantation (Saadeldin et al., 2024). These advancements show promise for toxicological studies, yet further research is needed to optimize their fine development and application (Papalia et al., 2007; Li et al., 2022).

7 Conclusion

The integration of advanced technologies such as organoids offers a promising approach to assess the reproductive toxicity of neurotherapeutic drugs. Organoids provide a physiologically relevant model that bridges the gap between traditional *in vitro* cell cultures and *in vivo* animal studies, allowing for more accurate assessments of drug effects on reproductive health. Despite their potential, current studies on neurotherapeutic drug-induced reproductive toxicity in organoid models are limited, highlighting the need for further research in this area. Addressing the current limitations of organoid technology, such as variability and maturity levels, is essential for understanding their full potential for toxicity screening and mechanistic studies. By advancing our understanding of the complex interactions between neurotherapeutic drugs and reproductive health, organoid modeling can lead to improved clinical management and reproductive risk mitigation strategies in drug development.

Author contributions

MA: Writing–review and editing, Writing–original draft, Conceptualization. J-SJ: Writing–review and editing, Writing–original draft, Validation, Supervision, Conceptualization. H-JK: Writing–review and editing, Writing–original draft, Supervision, Data curation. AA:

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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.

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The application of organoids in colorectal diseases

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Intestinal organoids are a three-dimensional cell culture model derived from colon or pluripotent stem cells. Intestinal organoids constructed *in vitro* strongly mimic the colon epithelium in cell composition, tissue architecture, and specific functions, replicating the colon epithelium in an *in vitro* culture environment. As an emerging biomedical technology, organoid technology has unique advantages over traditional two-dimensional culture in preserving parental gene expression and mutation, cell function, and biological characteristics. It has shown great potential in the research and treatment of colorectal diseases. Organoid technology has been widely applied in research on colorectal topics, including intestinal tumors, inflammatory bowel disease, infectious diarrhea, and intestinal injury regeneration. This review focuses on the application of organoid technology in colorectal diseases, including the basic principles and preparation methods of organoids, and explores the pathogenesis of and personalized treatment plans for various colorectal diseases to provide a valuable reference for organoid technology development and application.

KEYWORDS

colorectal cancer, organoids, tumor microenvironment, inflammatory bowel disease, gene editing

1 Introduction

"Colorectal diseases" is a collective term for benign and malignant diseases that occur within the colon, including colorectal cancer (CRC), inflammatory bowel disease (IBD), and intestinal infections. CRC is one of the most common colorectal diseases and may be related to changes in diet, increased obesity, environmental factors, and aging (Lu et al., 2021). CRC is the third most common cancer worldwide, accounting for 9.8% of all malignant tumors, and the second leading cause of cancer-related deaths, with a mortality rate of 9.2% (Sung et al., 2021). The World Health Organization has estimated the occurrence of 2.2 million new CRC cases and 1.1 million CRC-related deaths annually worldwide by 2030 (Ferlay et al., 2019).

IBD is a non-specific inflammatory disease of the digestive tract. It encompasses Crohn's disease (CD) and ulcerative colitis (UC) and is characterized by chronic inflammation leading to mucosal damage in the digestive tract (Chen et al., 2021). In 2017, the total number of patients with IBD worldwide reached 6.8 million (Ng et al., 2017), making this disease a massive burden on global public health services. The primary medical treatments for IBD include aminosalicylates, corticosteroids, biological agents, and immunosuppressants (Cai et al., 2021). Surgical intervention is required when medical treatment is ineffective or serious complications arise (Lamb et al., 2019). While biological

TABLE 1 Comparison of preclinical models in cancer research.

	2D cell lines	PDXs	Organoids
Establishment success rate	High	Relatively low	Relatively high
Maintenance	Easy	Difficult	Easy
Gene editing	Able	Unable	Able
Cost	Low	High	Relatively high
Expansion	Quick	Slow	Quick
Reproducibility	High	Moderate	Low
Representativeness	Low	Low	High
Tumor immune microenvironment	Unable to recapitulate	Partial recapitulation	Partial recapitulation
Tumor heterogeneity	Unable to recapitulate	Retain	Retain
Complexity	Low	High	High

agents can change the course of IBD, about one-third of patients fail to respond to them (Zhang et al., 2019). This indicates that existing treatment schemes have difficulty achieving satisfactory clinical efficacy.

Organoids are three-dimensional structures that self-assemble from stem cells, pluripotent cells, or tissue-specific cell types under *in vitro* culture conditions and have highly similar structures and functions to the source tissue or organ (Lancaster and Knoblich, 2014). In 2009, Sato et al. (2009) used leucine-rich repeat-containing G protein-coupled receptor 5 (*Lgr5*)$^+$ small intestinal stem cells to cultivate tissues with intestinal crypts and villi structures, creating a precedent for the organoid research field. This microenvironment biomimetic cell culture method has since been applied to more types of cell culture, and various patient-derived organoids (PDOs) have been successfully constructed (Barker et al., 2010; Sato et al., 2011; Rossi et al., 2018; Nikolaev et al., 2020).

PDOs can more faithfully simulate the biological behavior of tissues *in vivo* and have a more stable genome than traditional two-dimensional (2D) cell culture models. In addition, PDOs are easier to culture, with short construction times and high success rates, and can facilitate cell transfection and high-throughput screening more efficiently than patient-derived tumor xenograft (PDX) models (Table 1) (Bleijs et al., 2019; Tuveson and Clevers, 2019). Normal colon and tumor organoids can be cultured from specimens from patients with CRC with a success rate of over 90% (Van De Wetering et al., 2015). Even after long-term *in vitro* culture, organoids retain the original tumor tissue characteristics, gene expression profile, and metastatic potential *in vivo* (Jensen et al., 2023). Organoids effectively simulate the microenvironment of multiple cell interaction types in tissues *in vivo* and can reflect the tissues' physiological and pathological conditions (Li and Izpisua Belmonte, 2019). Therefore, organoids are suggested as a source of new ideas and methods for diagnosis, drug screening and development, and gene therapy for colorectal diseases.

2 Application of organoids in CRC

Molecular genetic studies have shown that CRC is a highly heterogeneous tumor that arises from various genetic variants via two major pathways: chromosomal instability and microsatellite instability (MSI) (Li et al., 2021). Different molecular subtypes of CRC have different drug sensitivities. However, a few patients are resistant to first-line CRC treatments. Therefore, individualized precision therapy is the key to treating advanced CRC (Keum and Giovannucci, 2019).

Previous *in vitro* studies on CRC mainly used 2D tumor cell lines and PDX models (Mouradov et al., 2014; Inoue et al., 2019). The 2D model can neither simulate tumors' spatial structure and heterogeneity *in vivo* nor reflect the cell interactions in the CRC microenvironment. While PDX models can strongly simulate the structure, heterogeneity, and physiological environment of tumors, the immune-deficient mice used to create them lack normal immune function and cannot be used for tumor immunity-related research and drug development (Neto et al., 2023). Unlike traditional models, organoid models derived from cancer tissues retain the molecular and biological characteristics of the malignant tissues (Mao et al., 2023). The organoid model provides a unique platform for studying tumors' biological characteristics, mechanisms of development and progression, drug sensitivity, and personalized therapy based on the mutated genes.

2.1 Organoid models for CRC

CRC organoids are generally constructed from tumor specimens or biopsies obtained by surgical resection (Figure 1), with a success rate of 60%–90% (Dijkstra et al., 2018). After enzyme treatment, the tissue can be embedded in Matrigel for suspension culture or cultured at a gas-liquid interface. To construct CRC organoids, epidermal growth factor (EGF), Wnt pathway agonist R-spondin 1 (RSPO1), and bone morphogenetic protein inhibitor noggin (NOG) must be added to replace the missing AChE-related signaling molecules. In addition, nicotinamide, transforming growth factor-beta (TGF-β) inhibitor a83-01, p38 mitogen-activated protein kinase (MAPK) inhibitor sb202190, and prostaglandin E2 can make the culture conditions suitable for the long-term growth of human primary colorectal adenocarcinoma (Van De Wetering et al., 2015). Organoid construction efficiency is reported to be improved by adjusting the medium composition and oxygen concentration (Fujii et al., 2018).

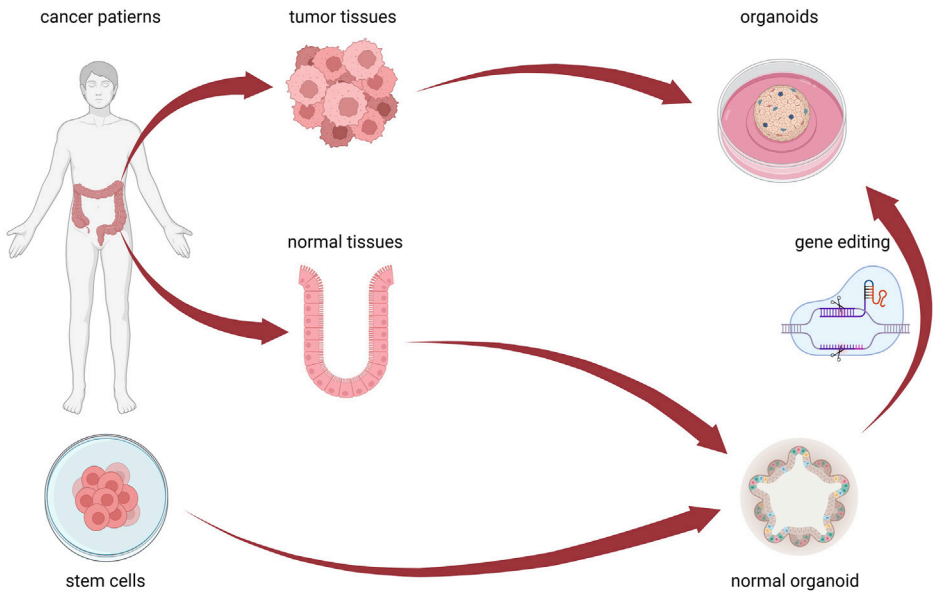

FIGURE 1
CRC organoid cultivation method. Patient-derived tumor-like organoids can be isolated and cultured from cancer tissue. Normal intestinal organoids can be isolated and + cultured from normal tissues, induced by stem cells to differentiate, and transformed into cancerous organoids through gene editing technology. Figure 1 was created with BioRender.com (accessed on April 4th, 2024).

In addition to using patient tumor tissue samples, CRC organoids can be constructed using gene editing technology to introduce specific gene mutations or modifications into stem cell-derived organoids to simulate genetic variation in CRC (Figure 1). APC Wnt signaling pathway regulator (*Apc*), transformation-related protein p53 (*Trp53*/*Tp53*), Kirsten rat sarcoma viral oncogene homolog (*Kras*), and SMAD family member 4 (*Smad4*) gene mutations were successfully introduced into the colon organoids of wild-type mice via short hairpin RNAs, biologically transforming them into invasive adenocarcinoma (Li et al., 2014). Specific mutated genes were introduced into normal colon organoids using clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) gene editing technology, revealing the role of EGF, Wnt, and TGF-β signaling pathways in the malignant transformation of the colon epithelium (Roper et al., 2017). After mutating *KRAS*, *APC*, *TP53*, and *SMAD4*, human intestinal organoids could grow in culture without the presence of stem cell niche molecules, and the p53 stabilizer nutlin-3 allowed them to exhibit the biological characteristics observed in invasive tumor tissues *in vivo* (Drost et al., 2015).

Utilizing organoids as preclinical models revealed the functional implications of sequential oncogenic mutations for promoting tumor proliferation, migration, and metastatic colonization in CRC (Fumagalli et al., 2017). The CRC development process was simulated by introducing specific mutations into human stem cell-derived organoids using CRISPR technology, uncovering the underlying mechanisms and providing crucial insights for developing related treatment methods (Drost et al., 2017). These studies improve the understanding of heterogeneous tumor genotypes and are of great significance for their personalized treatment (Schütte et al., 2017).

2.2 Application of organoids in the tumor microenvironment

The tumor microenvironment (TME) is a dynamic space within and around the tumor that largely determines its heterogeneity and plasticity (Almusawi et al., 2021). The conditions under which these microenvironments are reproduced in organoids are crucial for studying CRC. Tumor organoids typically consist of cancer cells derived from epithelial tissues, and establishing a TME in these organoids relies on artificial construction methods. Some CRC types, such as high MSI (MSI-H), B-Raf proto-oncogene, serine/threonine kinase (*BRAF*) mutant, and mucinous adenocarcinoma, depend highly on the TME, making it difficult to construct organoids successfully (Li et al., 2020). Therefore, the advanced coculturing of tumor-associated fibroblasts, immune cells, and other TME components with organoids is attractive.

Cancer-associated fibroblasts (CAFs) are a major TME component. They secrete stimulatory signals to support tumor development, suppress immunity, and promote drug resistance. Coculturing CRC-PDOs with two CAF types demonstrated that inflammatory CAFs promoted the epithelial-mesenchymal transition of CRC, while tumor-associated myofibroblasts reversed this effect (Mosa et al., 2020). Coculturing patient-derived CAFs with CRC-PDOs revealed that the tumor proliferated continuously without the addition of common growth factors to the PDO culture. Moreover, gene expression profiling and enrichment analysis of the model with TME components found that the immune response-related pathways missing in the non-coculture model were reactivated, showing that introducing TME components improved the simulation of the model (Luo et al., 2021).

Organoids play a crucial role in epigenetics. Preclinical studies of organoid and xenograft models have shown that DCAF1-mediated EZH2 phosphorylation plays an important role in gene reactivation in CRC cells (Ghate et al., 2023). The study of organoids in CRC also revealed that the absence of H4K20me3 mediated by Suv4-20h2 promotes the development of right CRC tumors through chromatin compaction (Boonsanay et al., 2023). In addition, organoid research uncovered the role of key molecular mediators. For example, SOX9 promotes stem cell activity and hinders normal differentiation in the development of CRC (Liang et al., 2022). Nuclear TYRO3 receptor tyrosine kinase molecular mediators such as BRD3 and MMP-2 play important roles in the metastatic process of CRC (Hsu et al., 2023).

The recent emergence of immunotherapy has advanced tumor-specific immunological *in vitro* models for patients with cancer. A personalized organoid platform for patients was constructed by coculturing organoids with homologous peripheral blood lymphocytes, and tumor-responsive T-cell were successfully enriched from the peripheral blood of MSI-H-type patients, confirming their cytotoxicity against homologous PDOs (Dijkstra et al., 2018). This finding enables the dynamic evaluation of individualized immune therapy efficacy for patients under minimally invasive conditions and offers the potential to utilize peripheral blood for adoptive T-cell therapy.

Chimeric antigen receptor (CAR) cell therapy holds great promise for microsatellite-stable CRC with weaker immunogenicity. In 2019, Schnalzger et al. (2019) reported a coculture platform of CRC organoids with CAR-NK cells, which allowed for the dynamic and quantitative monitoring of CAR-mediated cytotoxicity. Their findings suggested that the targeted effect of CAR cells on tumor-specific antigens enabled them to express tumor antigen-specific cytotoxicity even with trace amounts of tumor antigen expression or in complex microenvironments.

Furthermore, a previous study reported an air-liquid interface method for culturing mechanically dissected tumor tissue (Neal et al., 2018). This method constructed an organoid-like model containing the intrinsic tumor stroma, allowing the coculturing of tumor cells with naturally embedded immune cells. The tumor-infiltrating lymphocytes (TILs) within the model accurately retained the T-cell receptor repertoire of the original tumor. This organoid-like model simulated anti-programmed cell death 1 (PDCD1/PD-1) immunotherapy and exhibited tumor antigen-specific TIL activation and cytotoxic responses consistent with those *in vivo*. Moreover, this model successfully simulated the intrinsic immune components of the TME rather than peripheral blood immune cells, which are related to immune checkpoint inhibitors.

Organoids can reproduce the heterogeneity and microenvironment of tumors, laying the foundation for high-throughput drug sensitivity screening, personalized precision therapy, and further research on immunotherapy.

2.3 Application of organoids in anti-tumor drug screening

In recent years, new therapeutics, such as targeted and immunotherapeutic drugs, have improved patients' prognoses. However, due to tumor heterogeneity and the close relationship between patients' individual differences and drug efficacy, some patients still do not benefit from existing treatments (S et al., 2022). In addition, there is a gap between the current commonly used 2D culture tumor cell model and the cell characteristics of the original tumor, making it difficult to identify suitable drugs for personalized medication and new drug development (Hay et al., 2014). The organoid model has advantages in predicting the sensitivity of anti-tumor drugs over the classic 2D method of drug screening with CRC cell lines (Castro et al., 2021). Tumor cells within the same tumor can have different genetic and phenotypic characteristics, making treatment effective on some tumor cells but not others (Burrell et al., 2013). Compared to 2D tumor cell lines and PDX models, organoids can effectively simulate the TME *in vivo* and form a basis for high-throughput drug screening (Horvath et al., 2016).

In 2015, Van De Wetering et al. (2015) reported the first successful application of PDOs for high-throughput drug screening for CRC. They screened 83 drugs using a CRC organoid library from 20 patients and found that RAS-mutant organoids were insensitive to EGF receptor (EGFR) inhibitors. Vlachogiannis et al. (2018) reported the successful construction of a PDO model library using metastatic CRC samples from 16 patients. With this model, the therapeutic efficacies of regorafenib and cetuximab could be predicted with a sensitivity of 100% and a specificity of 93%. Yao et al. (2020) cultured 80 CRC organoids and assessed their sensitivity to radiotherapy and chemotherapy. Varinelli et al. (2024) cultured 12 CRC peritoneal metastases-derived organoids and demonstrated their utility in evaluating treatment plans at the patient level. These studies successfully connected cancer genetics with clinical trials, addressed the limitations of cell line-based and PDX models, and demonstrated that CRC organoids could be *in vitro* models for screening drugs and advancing precision medicine.

Schütte et al. (2017) tested the efficacy of 16 clinical drugs on organs derived from patients with CRC. They found that 14 genes, including regulator of G protein signaling 4 (*RGS4*), brain abundant membrane attached signal protein 1 (*BASP1*), and insulin-like growth factor 2 (*IGF2*), were associated with resistance to EGFR inhibitors and could serve as markers of insensitivity to EGFR inhibitors. The simultaneous blockade of the KRAS signaling pathway overcame resistance to targeted therapy of the MAPK pathway in metastatic CRC (Verissimo et al., 2016). In addition, organoid technology could be used to culture normal colorectal tissue from patients' tumors, screen anti-tumor drugs, reduce damage to normal cells, and reduce toxic and side effects (Vlachogiannis et al., 2018). These results suggest the potential value of using PDO models for the preclinical evaluation of anticancer inhibitors.

Organoid chip technology could be used for drug screening. An organoid chip is a microfluidic system that can reproduce three-dimensional (3D) structures and cell-cell and cell-material interactions within tissues *in vitro* (Low et al., 2021). Combining tumor organoid microfluidic chips and various cell sensors enables the monitoring of tumor cell status. Organoid chips could be used as an efficient, high-throughput tumor drug screening platform (Sun et al., 2019). Carvalho et al. (2019) developed a CRC microfluidic chip and successfully constructed a vascular support network with human colon microvascular endothelial cells, successfully simulating the correlation between drug concentration gradient and therapeutic effectiveness. Wang et al. (2020) pioneered a

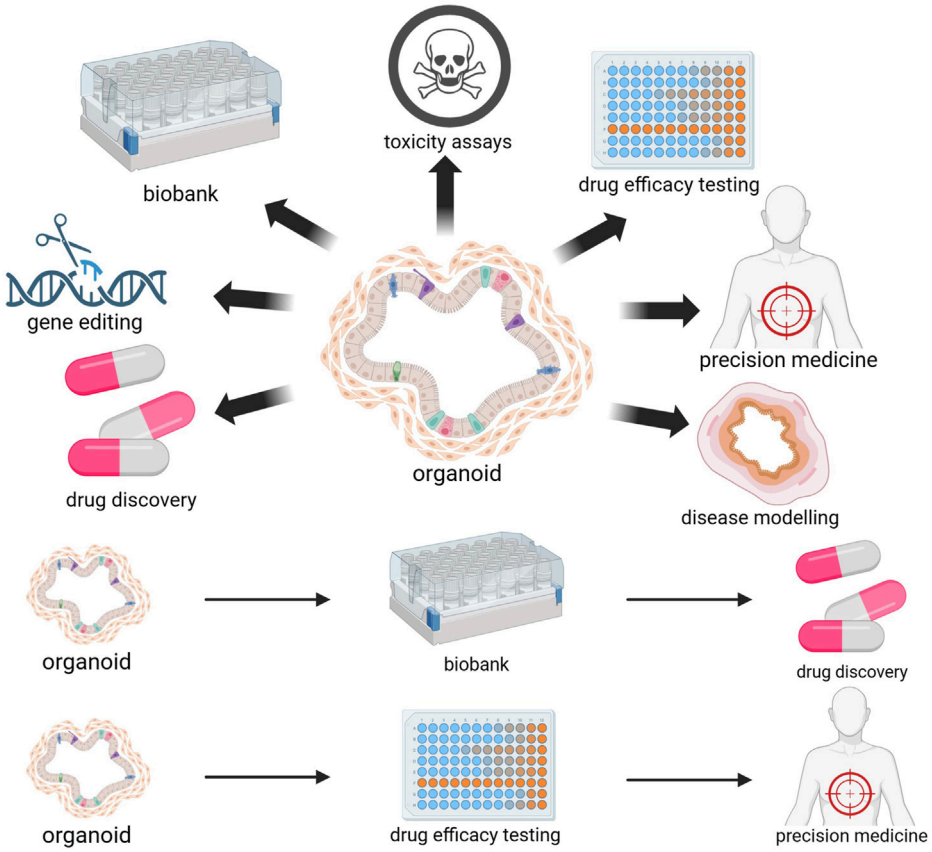

FIGURE 2
Biomedical applications of organoids. The utilization of tumor organoids derived from patients enables the prediction of individualized drug responses and personalized treatment outcomes. The freezing of organoids enables the establishment of organoid biobanks. Organoids are an ideal model for drug screening and toxicity testing and can be used for drug development. Organoids can also serve as disease models and undergo gene editing to promote research on CRC pathogenesis and physiological pathology. Figure 2 was created with BioRender.com (accessed on April 4th, 2024).

non-contact model system combined with digital sensing technology to non-invasively monitor CRC organoid proliferation and metabolism. Aleman and Skardal (2019) constructed a multi-site metastatic tumor chip covering CRC, liver, lung, and endothelial cells and other organoids that were interconnected by circulating fluid and used fluorescence imaging technology for cell tracking. The above-mentioned studies provide more efficient models for advancing and evaluating anticancer medications.

The application of tumor organoids has shown great potential in overcoming the challenges caused by tumor heterogeneity and individual differences. Organoids have shown significant value in predicting drug reactions and enabling high-throughput drug screening, providing a valuable platform for efficient drug application in CRC (Figure 2).

2.4 Application of organoids in personalized medicine

Personalized medicine aims to tailor treatment options for patients according to their specific genomics and metabolomics (Hamilton et al., 2021). The organoid model is close to the patient's physiological microenvironment, allowing for a more precise estimation of their drug reactions (Li et al., 2022). A prospective clinical study (Ooft et al., 2019) investigated the application of PDOs of CRC metastatic tumors to identify patients who failed to respond to standard chemotherapy regimens. Its results showed that PDOs accurately predicted the clinical responses of patients receiving irinotecan treatment. PDOs can provide accurate response prediction and guidance for personalized treatment. Ganesh et al. (2019) collected tumor tissues from patients with CRC at different stages and performed organoid culture. They also analyzed the clinical response of each patient to clinical chemotherapy or radiotherapy. They found a strong correlation between the projected outcomes from organoids and the clinical response observed in patients after treatment.

A prediction model was developed to analyze the radiotherapy response data of patients using a machine-learning algorithm (Park et al., 2021). When applied to 33 patients diagnosed with rectal cancer, the prediction accuracy of the radiosensitivity model of patient-derived tumor organoids was above 89%.

Tailored treatment plans could also be developed for patients by studying the impact of different treatment plans on organoids. Schwank et al. (2013) successfully applied CRISPR-Cas9 technology to human-derived organoids. They used this technology to repair the F508del mutation in the CF

transmembrane conductance regulator (*CFTR*) gene commonly found in patients, reinstating CFTR function in intestinal organoids. Geurts et al. (2020) also successfully corrected *CFTR* mutations using CRISPR-based technology and achieved the functional restoration of CFTR in intestinal organoids. Indeed, organoid technology can help treat CRC tumors by testing single or combination treatment plans in PDO models to determine the most effective plan for each patient and achieve personalized treatment (Figure 2).

2.5 Organoid biobanks

Organoid biobanks can provide data for drug development and contribute more to personalized and regenerative medicine than PDX models (Gunti et al., 2021). Van De Wetering et al. (2015) established the first CRC organoid biobank in 2015, significantly promoting genomic and functional research on organoids at the patient level. Another tumor tissue organoid biobank was created using 55 patients with CRC, shedding light on the functional connections and differences between tumors' genetic variation, ecological requirements, and biological phenotypes (Fujii et al., 2016). Another biobank of colorectal organoids derived from samples from 41 patients, including normal colon organoids derived from adjacent normal tissues, was established with an approximately 77% success rate (Ganesh et al., 2019). A biobank consisting of 80 colorectal tumor organoids was also successfully constructed, demonstrating the accurate prediction of neoadjuvant radiotherapy and chemotherapy efficacy for locally advanced CRC (Yao et al., 2020). An organoid biobank offers a collection of cancer organoid cultures encompassing the intricacies of diverse tumor subtypes (Zhou et al., 2021). This significant advancement greatly facilitates progress in novel drug development and screening processes (Figure 2).

3 Application of organoids in IBD

IBD pathogenesis is related to genetic predisposition, immune dysfunction, intestinal epithelial mechanical barrier damage, intestinal microbiota imbalance, and stimulation by environmental factors (Le Berre et al., 2023). As one pathogenic mechanism of IBD, intestinal epithelial mechanical barrier damage is interrelated with several other pathogenic factors and contributes significantly to IBD pathogenesis (Figure 3) (Khare et al., 2019). A genomic association study on IBD development identified some genes, such as innate immunity activator (*INAVA*/*C1orf106*), ring finger protein 186 (*RNF186*), and hepatocyte nuclear factor 4 alpha (*HNF4A*) as associated explicitly with maintaining epithelial barrier integrity (Graham and Xavier, 2020). Impairment of the intestinal epithelial barrier is a pivotal element of IBD pathogenesis. However, colorectal epithelial cell lines cannot fully recapitulate the heterogeneity of the intestine (Onozato et al., 2020). Moreover, their ability to reproduce the pathological and physiological characteristics of IBD is limited (Kotla and Rochev, 2023). Animal models of IBD are expensive, have long cultivation cycles, and cannot recapitulate human physiological characteristics (Schulte et al., 2019). Therefore, there is a pressing need for novel *in vitro* models to advance the understanding of IBD's pathogenesis and develop more effective treatment strategies.

The composition and arrangement of intestinal organoid cells are similar to the structure of intestinal epithelium, and they can be continuously passaged and cultured *in vitro* (Sato et al., 2009). Their chromosome number and expression profile are also highly consistent with those of their source (Dotti et al., 2017). Cultivating intestinal organoids requires a small amount of tissue, and both endoscopic biopsy and surgical specimens can be used as tissue sources. Moreover, the mucosa of both inflammatory and non-inflammatory sites in the intestine can form intestinal organoids *in vitro* (Liu et al., 2023). These results indicate that gut-like organs hold great promise as reliable instruments for studying IBD pathogenesis and drug screening.

3.1 Construction of organoid models for IBD

Intestinal organoid culture medium primarily consists of extracellular matrix (ECM) gel and culture medium. The ECM gel mainly provides mechanical support while transmitting molecular signals required for cell growth (Rezakhani et al., 2021). The culture medium is categorized into expansion and differentiation media. The expansion medium contains many growth factors essential for the proliferation of colonic stem cells and is used for the long-term cultivation and amplification of colonic organoids (Almeqdadi et al., 2019). The differentiation medium is based on the expansion medium but lacks components such as Wnt family member 3A (WNT3A). It is used to induce the transformation of stem cells into diverse terminally differentiated colonic epithelial cell lineages (Flood et al., 2023).

There are currently two well-established methods for constructing intestinal organoids: one involves using adult stem cells (ASCs) (Wallach and Bayrer, 2017) and the other entails directing the differentiation of induced pluripotent stem cells (iPSCs) into intestinal organoids (Tsuruta et al., 2020). Essentially, they recreate the *in vitro* microenvironment of the colon epithelium, facilitating the proliferation and specialization of cells found in the colon, resulting in the development of organoid structures comprised of a comprehensive range of colonic epithelial cell lineages (Zachos et al., 2016).

The construction of colonic organoids based on ASCs involves initially collecting colonic tissue specimens through surgical or endoscopic procedures, followed by the subsequent isolation and purification of colonic $LGR5^{+}$ stem cells. Next, these cells are combined with an ECM and multiple crucial growth factors, which support the continuation of the ASCs' ability to self-renew and undergo differentiation (Sato et al., 2011). The construction of colonic organoids from iPSCs necessitates emulating embryonic gut development *in vitro*. First, iPSCs are induced to differentiate into a definitive endoderm layer. This is then directed to differentiate into posterior endoderm and subsequently expanded to produce colonic organoids (Múnera et al., 2017).

Dotti et al. (2017) successfully constructed a UC organoid model by culturing colon tissue samples from patients with UC *in vitro*. They found that it maintained genetic and biological characteristics highly consistent with those of the body tissue during long-term

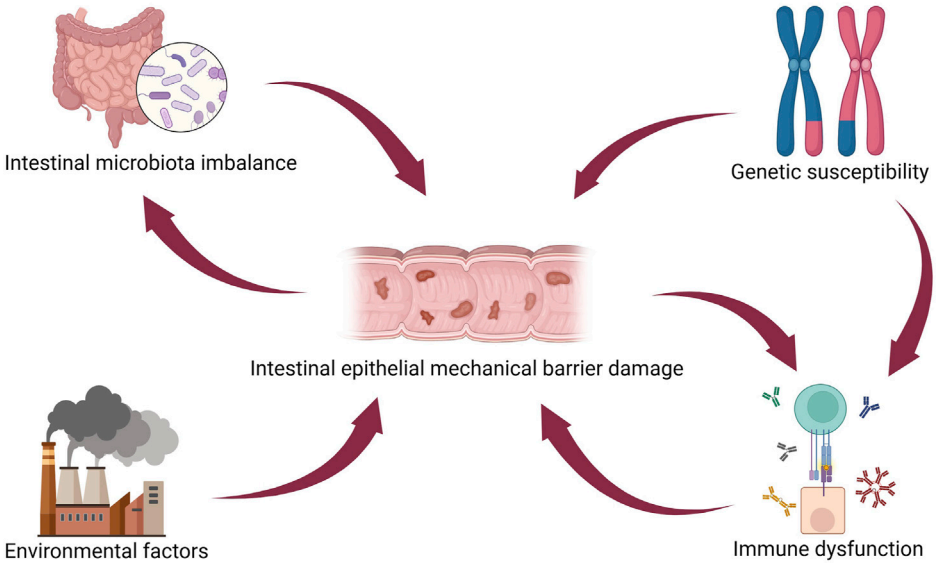

FIGURE 3
IBD pathogenesis. Intestinal epithelial injury directly interacts with several other pathogenetic mechanisms in IBD, such as intestinal microbiota imbalance, genetic susceptibility, environmental factors, and immune dysfunction. Figure 3 was created with BioRender.com (accessed on April 4th, 2024).

culture and passage. Colonic organoids constructed *in vitro* are highly similar to colonic epithelial tissue regarding cellular composition, tissue architecture, and specific functions (Zachos et al., 2016). Colonic organoids can effectively recapitulate the characteristics of colonic epithelial tissue in an *in vitro* culture environment.

3.2 Application of organoids in IBD pathogenesis

With their pluripotency, gene specificity, and structural polarity, intestinal organoids are progressively becoming a cellular experimental platform for IBD. Screening crucial pathways and transcriptional mechanisms driving epithelial dysfunction in patients with IBD within intestinal organoids is pivotal in elucidating IBD pathogenesis (Roh et al., 2019). Ishibashi et al. (2018) demonstrated that crypt stem cells derived from the diseased mucosa of patients with UC exhibited long-term differential transcriptional characteristics within organoids. They found that the antimicrobial peptide C-type lysozyme (*LYZ*), aquaporin 8 (*ACP8*), and transmembrane mucin 12 (*MUC12*) were downregulated, potentially leading to functional defects in colonic mucosal epithelial cells and contributing to the persistence of UC.

Sarvestani et al. (2018) performed immunohistochemical and next-generation sequencing analyses on an organoid model of UC constructed *in vitro*. Their findings revealed a remarkable level of agreement between the genomic and proteomic characteristics of the organism and this particular model. Single-cell sequencing of CD-derived intestinal organoids showed a notable disruption in the expression of *LYZ*, an antimicrobial peptide. Furthermore, there were distinct variations in the expression of markers associated with stem cells (Suzuki et al., 2018).

Hammoudi et al. (2022) found that mucosal T-cell originating from the same individual could directly trigger the death of epithelial cells. There was a direct association between T-cell infiltration in organ samples and epithelial cell death, which could be inhibited by blocking the lymphocyte-epithelial cell interaction through integrin subunit alpha E (ITGAE/CD103) and killer cell lectin-like receptor K1 (KLRK1/NKG2D) blocking antibodies.

Rees et al. (2020) discovered that the endoplasmic reticulum stress pathway is dysregulated in colonic organoids derived from both UC and CD. This dysregulation may increase the functionality of Toll-like receptor 5 (TLR5), elevating the secretion of interleukin 8 (IL8) and persistently activating peripheral dendritic cells, ultimately resulting in mucosal autoinflammatory responses.

3.3 Application of organoids in IBD treatment

IBD treatment mainly relies on drugs. Intestinal organoids can comprehensively simulate the internal environment and are highly similar in structure and function to the intestinal epithelium; thus, they can be used for drug detection. The clinical conversion success rate of IBD drugs in the gut organoid model has significantly increased (Kopper et al., 2021). Organoid models allow for evaluating the efficacy of conventional drugs and investigating novel drugs for treating IBD (Lacombe et al., 2022). Corticosteroids are commonly used to treat IBD (Pithadia and Jain, 2011). Using a confocal microscope, researchers detected the presence of fluorescein isothiocyanate-dextran 4 (FD4) infiltrating the lumens of intestinal organoids (Xu et al., 2021). Further research found that treating IBD organoids with corticosteroid prednisolone significantly reduced FD4 infiltration in the lumens and

reduced the expression of inflammatory factors, indicating that corticosteroids are effective in treating IBD.

Treating intestinal organoids with tumor necrosis factor (TNF)-α resulted in the internalization and abnormal degradation of E-cadherin and decreased tight junction protein 2 (TJP2) levels (Khare et al., 2019). Treatment with 5-aminosalicylic acid (5-ASA) or azathioprine (AZTP) restored E-cadherin and TJP2 levels on the cell membrane to normal. These findings confirmed the ability of AZTP and 5-ASA to treat IBD, which is consistent with previous clinical research results (Swidsinski et al., 2007).

Lee et al. (2021) found in a study of 3D patient-derived intestinal organoids from CD patients that the reconstruction rate and cell viability were significantly impaired following TNF-α exposure. Kawamoto et al. (2019) examined the impact of infliximab, an anti-TNF-α medication, on intestinal organoids. They found that cotreatment of organoids with infliximab and TNF-α did not significantly affect their vitality or morphology but notably reduced ubiquitin D (*UBD*) expression, suggesting that infliximab has anti-inflammatory effects in treating IBD. Lloyd et al. (2020) added the macrolide antibiotic clarithromycin to colon organoids from healthy individuals. They found that clarithromycin had antibacterial effects and inhibited intestinal dermatitis.

In addition to medication, autologous intestinal transplantation after the *in vitro* expansion of self-derived intestinal organoids is a promising treatment approach for intractable ulcers and other disease manifestations in patients with IBD. Compared to conventional drug therapy, organoid mucosal therapy modulates the stem cell microenvironment at the lesion sites and promotes ulcer healing (Okamoto et al., 2020). Yui et al. (2012) found that colonic organoids cultured *in vitro* and then transplanted into a mouse model with acute UC induced by dextran sodium sulfate (DSS) could precisely reach the affected colonic epithelium and effectively restore the damaged tissue. They confirmed that expanding colonic stem cells *in vitro* and reintroducing them into the body could promote colonic epithelial regeneration and cure colonic mucosal damage. Watanabe et al. (2022) demonstrated that colonic organoids transplanted via the rectum into mice with UC could effectively reach and repair the damaged intestinal epithelium. Fordham et al. (2013) transplanted mouse small intestinal organoids into a mouse colonic injury model and found that they differentiated into colonic-like epithelial tissue, indicating that intestinal organoids possess immature cells that can adapt to the transplantation site by altering their phenotype. In a separate study by Sugimoto et al. (2018), normal human colonic organoids were successfully transplanted into the colons of immunodeficient mice, where they retained the characteristics of human colonic tissue and remained viable. These findings suggest that human intestinal organoid transplantation holds tremendous potential for treating IBD.

4 Application of organoids in intestinal infection

With improved sanitation conditions and advances in medical care, there has been a significant decrease in outbreaks of gastrointestinal infectious diseases and a noticeable reduction in mortality rates associated with infectious diarrhea (Meisenheimer Es et al., 2022). Intestinal organoid culture systems faithfully recapitulate the environmental conditions of the intestinal epithelium, allowing us to further explore the complex intestinal microbial ecosystem (Hentschel et al., 2021).

4.1 Construction of intestinal infection organoid models

By co-cultivating intestinal organoids with pathogens, it is possible to generate organoids that simulate intestinal infections. These organoids have an inward-facing layer of epithelial cells, requiring the microinjection of microorganisms into the organoid lumen. This method has been successfully used to construct various organoid models of intestinal infection, including ones for enterohemorrhagic *Escherichia coli* (Pradhan and Weiss, 2020), *Salmonella enterica* serovar Typhi (Geiser et al., 2021), and *Cryptosporidium parvum* (Heo et al., 2018) infections (Figure 4). Moreover, organoids can sustain the growth of microbial communities from human fecal isolates within the cavity. However, the workload required for the intraluminal microinjection of organoids is high, making it difficult to control the diversity of infections (Williamson et al., 2018).

An inverted, apical-out organoid culture model has been developed to address these challenges. The methods for its construction involve removing the ECM and culturing the organoids in low-adherent plates, resulting in polarity inversion while maintaining functional and barrier integrity (Figure 4) (Co et al., 2019; Co et al., 2021). However, these methods result in the inability to access the basolateral side and still do not address the issue of infection diversity. An alternative strategy that overcomes these limitations involves culturing individual cells derived from organoids in a single layer (Roodsant et al., 2020). This method entails enzymatically dissociating the organoids into single cells, which are then seeded onto transwell inserts, ensuring the retention of the complexity of intestinal epithelial cells while establishing proper polarity and barrier formation. This approach enables access to both the apical and basal sides of the intestinal epithelium.

The single-layer model derived from human intestinal organoids can recapitulate the different intestinal cell populations, making it a valuable tool for studying specific infections in the gut (Figure 4). This method has been successfully used to study infections caused by pathogens such as *Shigella*, *Salmonella*, and pathogenic *E. coli* (Vandussen et al., 2015; Nickerson et al., 2021). Organoid technology can also be used to study viral invasion and intracellular replication. Notably, in the case of previously uncultivable viral agents such as norovirus (Ettayebi et al., 2016), human intestinal organoids have been crucial in constructing *in vitro* models. These models have been improved to investigate the interactions between viruses and intestinal cells and the host response to viral infections.

4.2 Application of organoids in intestinal infection

Gut organoid models can reveal the infection mechanism of intestinal pathogens. Saxena et al. (2016) demonstrated that the human rotavirus RV1 vaccine strain induced the expansion of small intestinal organoids through the action of the enterotoxin nonstructural protein 4 (NSP4) fragment, leading to a diarrheal

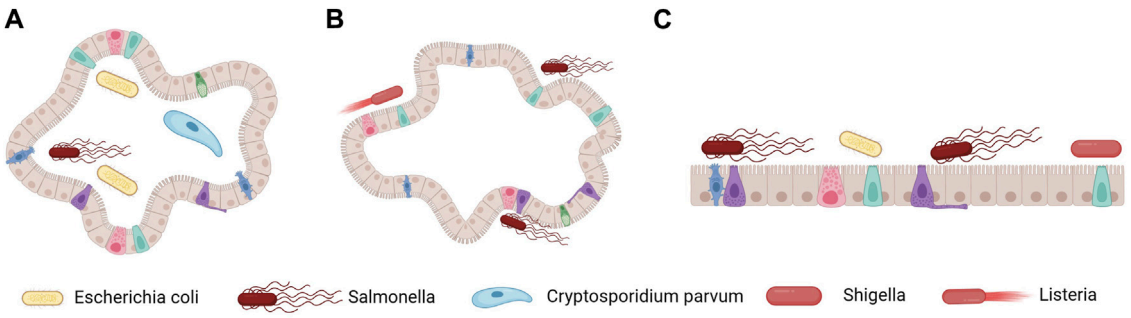

FIGURE 4
Methods for constructing organoid models of intestinal infection. **(A)** Microorganisms are introduced into the cavity of 3D organoids via microinjection, thereby achieving microbial epithelial interaction at the top. **(B)** The polarity of intestinal compounds is reversed, and microorganisms are added to the external medium. **(C)** Organoids are enzymatically dissociated to form 2D polarized monolayers, and microorganisms are added at the top or outer base. Figure 4 was created with BioRender.com (accessed on April 4th, 2024).

response resembling physiological conditions. The rotavirus evades the host's innate immune response by interfering with the nuclear factor kappa B (NF-κB) pathway in epithelial cells through nonstructural proteins 1 (NSP1) and 3 (NSP3), inhibiting the secretion of type III interferons after the activation of pathogen-associated molecular patterns (Saxena et al., 2017). Compared to traditional cell lines, such as the MA104 strain of rhesus monkey embryonic kidney cells and the CV-1 strain of African green monkey kidney cells, rotavirus demonstrates enhanced replication capacity in intestinal organoids.

Ettayebi et al. (2016) demonstrated that norovirus can utilize histo-blood group antigen receptors in the gastrointestinal tissue to exert its invasive effects when exogenous bile is added to the organoids. Drummond et al. (2017) observed that the specific cytotoxic effects of enterovirus type 11 could disrupt crypt structures, mislocalize tight junction proteins, and induce the release of inflammatory mediators. In contrast, no antiviral response was detected in intestinal organoids infected with coxsackievirus B and enterovirus type 71.

Intestinal organoids have also revealed the segment-specific and cell-specific characteristics of pathogen infections. For example, common pathogens causing childhood diarrhea, such as enteropathogenic *E. coli*, exhibit a stronger affinity for duodenum, ileum, and colon organoids (Holly and Smith, 2018). Additionally, various pathogens infect different cell types during the invasion process. Rotavirus primarily invades enterocytes and enteroendocrine cells (Saxena et al., 2016). Enterovirus type 11 cannot replicate in goblet cells, while enteric adenovirus type 5p preferentially infects goblet cells (Rajan et al., 2018). Using 3D confocal reconstruction techniques, Park et al. (2016) observed the entire process of *S. enterica* serovar Typhi-induced epithelial microvillus fold formation, intracellular bacterial replication, and necrotic cell shedding.

5 Application of organoids in regenerative medicine

The amount of tissue required for organoid cultivation is minimal, and most tissues can be obtained through minimally invasive procedures (Yi et al., 2021). The culture environment of organoids closely resembles human tissue and allows for large-scale *in vitro* cultivation, making organoid transplantation feasible (Bock et al., 2021). Tissue-specific ASCs within organoids can differentiate into relevant tissues or organs, enabling the direct induction of tissue regeneration during transplantation and maximizing the effectiveness of regenerative treatment (Choi et al., 2023). Organoids derived from ASCs exhibit a reduced likelihood of tumor development. Moreover, their direct transplantation at the injury site reduces their potential migration and dissemination to other organs. Using autologous cells in organoid therapy also decreases the likelihood of immune tolerance. Finally, most indications for organoid-based therapy can be treated with minimally invasive transplantation guided by endoscopy or ultrasound (Li et al., 2019).

Sugimoto et al. (2018) demonstrated that the transplantation of colon organoids into the inflamed intestines of mice induced by DSS and human-derived intestinal organoids into immunodeficient mice with mucosal damage both led to intact epithelial function and lineage tracing, confirming the excellent transplantability and high plasticity of the colon epithelium. Animal-derived decellularized ECM was found to be a suitable culture matrix for supporting organoids and *in vivo* transplantation, with modified organoid culture efficiency approaching that of matrix gel (Giobbe et al., 2019). Meran et al. (2020) developed allogeneic intestinal scaffolds, where donor-derived organoids were transplanted onto decellularized scaffolds to form repopulated grafts. These grafts maintained their luminal structures long after transplantation into mice. Okamoto et al. (2023) used a tissue-engineering approach with embryonic and iPSCs to generate human intestinal organoids, which can be combined with neural cells to gain intrinsic motility function.

Bioengineering studies on the vascular structures associated with organoids have also made it possible to transplant large tissue blocks (Grebenyuk and Ranga, 2019). Brassard et al. (2021) proposed creating a small intestine by combining 3D bioprinting technology with organoids. Using 3D bioprinting of human umbilical vein endothelial cells, mesenchymal stem cells, and intestinal organoids, they systematically fabricated centimeter-scale tubular intestinal epithelial tissue with a glandular-villus-like structure, connective tissue, and a vascular network.

Sugimoto et al. (2021) constructed functional small intestine-like colons using ileum-derived organoids, achieving the reconstruction of the entire small intestine by replacing colon epithelial tissue and providing a feasible strategy for treating short bowel syndrome. In a non-clinical study, Jee et al. (2021) found that colon organoid-based regenerative therapy effectively treated radiation proctitis and restored damaged colon epithelial structure and integrity. Therefore, intestinal organoids have opened up a new avenue for research in colonic regenerative medicine.

6 New technologies for organoid construction

6.1 Applications of CRISPR-Cas9 technology in organoids

Gene editing is an emerging molecular biology technique that involves artificially altering a specific gene locus to modify the expression characteristics of the target gene, facilitating the study of gene function (Huang et al., 2021). CRISPR-Cas9 is a novel gene editing technology that utilizes an RNA-mediated adaptive immune defense system first discovered in 1987 (Ishino et al., 1987). CRISPR-Cas9 mainly comprises the *Cas9* gene, an RNA-guided endonuclease, and the CRISPR sequence, which consists of multiple conserved repeat sequences and interval sequences with a regular pattern. The Cas9 nuclease can recognize and cleave complementary DNA strands specific to the target site under the guidance of the corresponding guide RNA (Doudna and Charpentier, 2014; Shalem et al., 2014; Manghwar et al., 2020).

CRISPR-Cas9 technology offers advantages over traditional gene editing techniques, such as high flexibility, cost-effectiveness, and ease of use, making it the most convenient and powerful tool for gene editing (Karimian et al., 2019). The advent of CRISPR-Cas9 technology significantly simplified the challenges associated with human gene editing, and it found wide applications in the organoid field. Gene modification techniques based on CRISPR-Cas9 can introduce arbitrary combinations of cancer gene alterations into normal organoids to design cancer organoid models (Tuveson and Clevers, 2019). Utilizing CRISPR-Cas9 technology to edit commonly mutated genes in CRC and introduce them into normal human colon organoids ultimately results in CRC models with different phenotypes (Matano et al., 2015). Fujii et al. (2015) simulated the early development and progression of CRC by combining organoids with CRISPR-Cas9 technology. Artegiani et al. (2020) described a CRISPR-Cas9-mediated homology-independent targeted integration technique in organoids that enabled the precise integration of exogenous DNA sequences into organoids. Fumagalli et al. (2017) simulated the evolution of colorectal adenoma-carcinoma *in vivo* by transplanting colon organoids containing different mutated genes, elucidating how gene alterations in the WNT, EGFR, TP53, and TGF-β signaling pathways contribute to the growth, migration, and metastasis of colorectal tumors. CRISPR-Cas9 provides an effective tool for studying organoids.

6.2 Application of 3D bioprinting technology in organoids

An emerging technique, 3D bioprinting technology involves fabricating *in vitro* 3D structural models using 3D printing technology, biological units, and biomaterials based on the functional requirements of living organisms (Liu et al., 2017). Traditional organoids are generated via stem cell expansion, specialization, and autonomous organization, lacking precise regulation over cell numbers, cell lineages, and the microenvironment (Mu et al., 2023). 3D bioprinting technology enables the construction of complex organoid structures through stable model building and multicellular-controlled organoid printing, allowing for the simultaneous printing of multiple cell components, ECM, and growth factors (Gong et al., 2021).

In 2020, Kim et al. (2020) proposed an organoid model based on 3D bioprinting technology, revealing the critical role of signaling crosstalk between tumor cells and stromal cells in controlling tumor plasticity. Chen et al. (2020) used 3D printing technology to fabricate a bio-scaffold and implant HCT116 human colon cancer cells, CAFs, and tumor-associated endothelial cells onto the scaffold, successfully constructing a 3D coculture colon cancer model. The 3D scaffold provided excellent support for the cells and helped maintain cell adhesion, proliferation, stemness, and vascularization. The activated stromal cells in the model exhibited high expression of various tumor-related factors and reshaped the ECM, while the tumor tissue showed transcriptomic characteristics highly similar to those *in vivo*.

Sbirkov et al. (2021) successfully constructed a novel 3D-printed model using Caco-2 human colon cancer cells. In this model, Caco-2 cells exhibited a realistic glandular-like histological morphology, and their RNA expression profile showed the upregulation of genes related to cell adhesion, hypoxia, and the EGFR/KRAS pathways and downregulation of genes related to cell cycle regulation. Kim and Kim (2020) described a novel bioprinting technique that uses cell-laden bio-ink composed of collagen and decellularized small intestine submucosa and successfully manufactured intestinal models with microvillus structures. These models exhibited physiological structures that closely resembled the intestine in terms of cell viability, alkaline phosphatase and aminopeptidase activity, permeability coefficient, and glucose uptake capacity.

6.3 Application of microfluidic devices in organoids

Microfluidic devices integrate sample preparation, reaction, separation, and detection into microchip platforms based on precision engineering, biomaterials, and tissue engineering. They enable the precise manipulation of tiny fluid flow through microchannels between different compartments and provide a technical platform for automated detection and analysis (Poenar, 2019). Microfluidic devices can be used to cultivate multiple cell types, organs, and tissues on the same platform, allowing for the precise control of component quantities, layouts, and spatial connections. When combined with techniques such as spheroids and organoids, microfluidic devices can construct complex and

precise *in vitro* 3D models with high throughput, customization, low sample volume, and high efficiency (Saorin et al., 2023).

Microfluidic devices facilitate the reconstruction of complex TMEs and the simulation of microvascular systems, overcoming the limitations of traditional organoid models (Lin et al., 2017). Using a microfluidic device, Sontheimer-Phelps et al. (2020) successfully constructed a human colonic chip with a normal colonic mucus layer structure and function. Rajasekar et al. (2020) described a novel microfluidic system and co-cultivated self-assembled vascular networks with colonic organoids, achieving better organoid growth under constant perfusion conditions. Carvalho et al. (2019) incorporated HCT116 human colon cancer cells into a matrix gel as a tumor core and successfully constructed a CRC microfluidic chip with a vascular support network using human colonic microvascular endothelial cells. Shin et al. (2020) reported a patient-specific 3D physiodynamic mucosal chip that simulated *in vivo* intestinal fluid dynamics and cocultured it with the gut microbiome, resulting in the disease-specific differentiation of intestinal organoids. These data indicate that microfluidic devices hold significant promise and offer immense potential in tissue engineering, pharmaceutical research, and individualized healthcare.

7 Challenges and future directions

Organoids precisely replicate organ architecture and functionality, encompassing diverse cell types, tissue arrangements, and cellular interactions. These miniature organ models can be cultivated from a small number of cells or tissue samples and serve as effective tools for disease modeling and drug screening. They also hold potential for therapeutic interventions by enabling the reversal of pathogenetic mutations responsible for mutation-induced diseases (Carvalho et al., 2023). Using patients' cells to construct organoids provides new possibilities for formulating personalized diagnosis and treatment programs (Betge and Jackstadt, 2023). Organoids have great potential in colorectal diseases. Using organoids for *in vitro* evaluation can influence clinical decision-making and improve the survival rate of patients. However, organoid technology is not yet fully mature and still faces many challenges.

The viability of organoid cultures is frequently hindered by the scarcity of viable cells in patient-derived specimens, posing a challenge to their successful establishment. Organoid culture techniques are still in the exploratory stages, with no consensus among experts regarding specific operational procedures (Li et al., 2020). Therefore, standardized protocols for organoid cultures are urgently needed. The high cost of growth factors and culture additives restricts the widespread adoption of organoid culture techniques. Moreover, adding multiple growth factors may induce genetic mutations, resulting in discrepancies between mechanistic exploration or drug sensitivity testing results and real-world scenarios (Lesavage et al., 2022). Despite their being valuable preclinical models for predicting drug efficacy, the full translation of organoids to clinical applications is challenging due to considerations such as drug side effects, underlying patient conditions, unconventional treatments, and medical ethics (Garreta et al., 2021).

Organoid technology offers immense potential for diverse applications across various fields. Organoids can be used to construct transplantable substitute organs, replacing diseased or damaged organs and providing temporary or permanent functional replacements, thereby improving patients' quality of life (Rossi et al., 2018). Moreover, constructing PDO models can provide a deeper understanding of disease progression, assess the effectiveness of different treatment approaches, and establish a foundation for personalized medicine (Qu et al., 2021).

8 Conclusion

Organoids have the advantages of an appropriate culture cycle, stable passage, and high-throughput drug screening capability. They have broad application prospects in modeling CRC development and progression mechanisms and evaluating clinical treatment efficacy. While some shortcomings and limitations remain, new technological developments will produce a new generation of organoid models that even more faithfully recreate the *in vivo* picture. In summary, as preclinical models, organoids hold great potential to expedite the translation of basic research into clinical practice and provide valuable evidence to guide the clinical treatment of intestinal diseases.

Author contributions

YLi: Writing–original draft, Writing–review and editing. DW: Writing–review and editing. YLu: Writing–review and editing. BT: Writing–review and editing. QL: Writing–review and editing. QF: Writing–review and editing. HZ: Writing–review and editing. JM: Writing–review and editing. JY: Writing–review and editing.

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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.

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Research progress on the application of organoids in gynecological tumors

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Organoids are *in vitro* 3D models that maintain their own tissue structure and function. They largely overcome the limitations of traditional tumor models and have become a powerful research tool in the field of oncology in recent years. Gynecological malignancies are major diseases that seriously threaten the life and health of women and urgently require the establishment of models with a high degree of similarity to human tumors for clinical studies to formulate individualized treatments. Currently, organoids are widely studied in exploring the mechanisms of gynecological tumor development as a means of drug screening and individualized medicine. Ovarian, endometrial, and cervical cancers as common gynecological malignancies have high morbidity and mortality rates among other gynecological tumors. Therefore, this study reviews the application of modelling, drug efficacy assessment, and drug response prediction for ovarian, endometrial, and cervical cancers, thereby clarifying the mechanisms of tumorigenesis and development, and providing precise treatment options for gynecological oncology patients.

KEYWORDS

gynecological tumors, organoid, ovarian cancer, endometrial cancer, cervical cancer, tumor models

1 Introduction

Cervical cancer (CC), endometrial cancer (EC), and ovarian cancer (OC) are known as the three major malignant tumors in gynecology, which are silent killers of women's lives and health worldwide. Inhibiting the occurrence and development of the three major malignant tumors in gynecology is a major challenge currently facing the medical field. Organoid is an emerging *in vitro* 3D modeling technology with the characteristics of reproducing the physiological and pathological features of the original tissues *in vivo*, which has shown good applicability in the fields of disease modeling, regeneration mechanism, and precision medicine. Therefore, further thinking about the integration of gynecological malignancies and organoids, with modeling approach as the starting point and drug efficacy assessment and drug response prediction as the endpoints for research, may help to provide a new strategy of targeted drug delivery for the treatment of gynecological malignancies featuring precision and personalization. Gynecological malignant tumors are major diseases that pose a serious threat to women's lives and health worldwide. Studies have shown that the incidence and mortality rates of common gynecological malignant tumors such as CC, EC, and OC have increased rapidly in recent years (Miller et al., 2019). Gynecological malignancies place a heavy medical burden on women's overall health, with uterine,

cervical, and ovarian cancers causing approximately 30,000 deaths per year in the United States (Ferlay et al., 2015). In the conventional treatment of gynecological malignancies, surgery plus chemotherapy is the treatment of choice for most patients. However, standard therapies are not applicable to every patient; therefore, there is an urgent need for effective methods to understand the tumor characteristics of patients to perform predictive analyses of tumors and provide more personalized and precise treatment options (Wahida et al., 2023).

Cell cultures and animal models are the experimental cornerstones of the medical field for the study of tissues, organs, and body physiology. However, their ability to accurately reflect human mechanisms *in vivo* is limited. Organoids are micro-organs with three-dimensional (3D) structures that can highly mimic the morphology and physiological functions of the source tissues and produce tissues with stable phenotypes, making them good models for clinical disease research (Semertzidou et al., 2020). As research continues to evolve, human-derived models have progressed from monolayer cell cultures to 3D organoids, and cultivating 3D organoids can be used to explore the mechanisms of disease occurrence and development. Tumor organoids have become the dominant experimental model for exploring the diagnosis and treatment of tumor diseases, and can be used to analyze the genetic or epigenetic basis, uncover drug resistance mechanisms, conduct drug screening, and individualize treatment for patients, so this direction is a breakthrough point, and it is hoped that it will enhance the prospects for personalized medicine based on the study of organoids of patient origin (Driehuis et al., 2020; Li et al., 2021; Shamshirgaran et al., 2021; Wu et al., 2021; Wang et al., 2023).

Previous studies have shown that organoids can reproduce the biological characteristics and tumor heterogeneity of primary tissues, making them a novel and reliable clinical model in the study of gynecological malignant tumors (Sachs et al., 2018; Kopper et al., 2019; Yoon et al., 2021). Kopper et al. established 56 organoid lineages from OC tissue and tested their sensitivity to commonly used platinum or paclitaxel drugs in OC treatment regimens. Their results showed that the HGS-3.1 organoid lineage was highly sensitive to drugs such as gemcitabine, carboplatin, and paclitaxel, while exhibiting resistance to drugs targeting the PI3K/AKT/mTOR pathway (Kopper et al., 2019). McDowell et al. evaluated the activity of artemisinin in primary OC organoids and found that artesunate treatment can induce G1 phase arrest, upregulate G1/S conversion, and reduce cell viability in OC cell models (McDowell et al., 2021). Artesunate combined with carboplatin and paclitaxel can enhance its effectiveness and enhance clinical efficacy (McDowell et al., 2021). Bi et al. established organoid models of EC and OC tissues from patient sources, tested the most used drugs, and found that these models reflected a series of sensitivities to platinum-containing chemotherapy and other related drugs (Bi et al., 2021). They successfully predicted the organoid postoperative chemotherapy and trastuzumab resistance of patients receiving neoadjuvant trastuzumab treatment before surgery, suggesting that organoids can be used as a preclinical platform for personalized cancer treatment in patients with gynecological cancer (Bi et al., 2021). Therefore, organoids not only play an important role in exploring signaling pathways, gene screening, and other mechanisms of tumor occurrence and development in the field of gynecological cancer but also provide a platform for mutation carcinogenesis modeling, drug screening, patient stratification, and drug response prediction. This fills the gap between basic research and clinical practice and helps with personalized treatment (Semertzidou et al., 2020).

In summary, it can be found that organoids have been widely used in the research of gynecological tumors. With this background, we reviewed common gynecological tumors such as OC, EC, and CC and analyzed the application research progress of organoids in gynecological tumors from the perspectives of modeling, drug screening, and drug evaluation to provide directions for future research.

2 Ovarian cancer organoids

Among all gynecological malignancies, OC has the highest mortality rate owing to high treatment resistance, long incubation period, and lack of effective treatment methods (Torre et al., 2018; Siegel et al., 2020). This requires preclinical models to summarize the histological, molecular, and pathophysiological characteristics of different subtypes of OC. Furthermore, OC is a heterogeneous disease composed of a series of subtypes, among which epithelial type is the most common. Given the significant heterogeneity of OC, the same treatment method can produce different clinical outcomes in individual patients (Kurman and Shih Ie, 2016). For example, poly ADP ribose (PARP) inhibitors are effective in patients with homologous recombination deficiency (HRD), but ineffective in patients without HR deficiency (Yang et al., 2021). Therefore, there is an urgent need for promising preclinical models to achieve precision medicine. Organoids, as models from patients, can address the heterogeneity of OC and simulate the progression of OC in patients, providing important insights for personalized treatment.

2.1 Modeling of OC organoids

At present, research has successfully developed various histological subtypes of organoids, including serous OC organoids, epithelial OC organoids, clear cell carcinoma, and endometrioid OC organoids (Nanki et al., 2020; Wan et al., 2023; Wu et al., 2023). By constructing an OC disease model, capturing the characteristics of histological cancer subtypes, replicating the mutation landscape of the primary tumor, and preserving the genomic map of the originating tumor, the usefulness of identifying genes related to OC progression can contribute to the study of disease etiology (Iwahashi et al., 2022). Among them, serous OC organoids are the current research focus. The following shows the modeling methods for ovarian cancer organoids with different tissue types.

2.1.1 Serous OC organoids

The matrix gel method is currently the main method for culturing serous OC organoids, with the main difference being the source of the extracted samples. Raab et al. extracted samples from patients with epithelial OC and cultured serous OC organoids using the matrix gel method. They found that restoring p53 function can inhibit the proliferation of advanced serous ovarian cancer

(HGSOC) cells, reduce chromosomal instability, and cause cell death (Raab et al., 2024). Iwahashi et al. extracted samples from 121 patients with wild-type p53 or mutant p53 high-grade serous OC and constructed OC organoids using the matrix gel method. They found that mutant p53 aggregates hindered the apoptotic function of wild-type p53 in recipient cells, and p53 aggregation inhibitors restored cell apoptosis in tumor organoids derived from patients carrying p53 aggregates, which is an appropriate p53 function (Iwahashi et al., 2022). The transmission of p53 aggregates is related to poor cancer prognosis and chemotherapy resistance (Iwahashi et al., 2022). Carvalho et al. drained ascites or pleural effusion from patients diagnosed with serous OC and cultured them into organoids using the matrix gel method. The study found that ligands and receptors of the PI3K-AKT pathway are important mediators for cancer-associated fibroblasts (CAFs) to communicate with other cancer cells (Carvalho et al., 2022). Compadre et al. constructed organoids from tumor biopsies or malignant ascites collected from patients with HGSOC by using the matrix gel method and showed that DNA repair protein RAD51 scores of platinum non-responding tumor organoids were significantly higher than those of platinum-responsive tumor organoids, and that the RAD51 foci were a potent marker of response to platinum-based chemotherapy and survival in OC (Compadre et al., 2023). Cao et al. cultured a high-grade plasmacytoid carcinoma organoid model that was highly similar to clinical OC tissues (Cao et al., 2023). Clinical OC tissues extracted from patients were used to culture the organoids by using the matrix gel method. After development of the organoids, they were passaged every 15 days, and the culture was successful when the fragments were rounded and showed features of the parental tumor within 7 days.

On the basis of the matrix gel method, some studies have attempted to improve the cultivation method or tissue source. Wang et al. quickly transferred fresh high-grade serous cancer tissue into the cell culture room after surgery and used matrix gel method to form organoids. The improvement in this study was the addition of 500 μL advanced DMEM/F12 medium containing specific growth factors along with 10 mmol/L niacinamide, and it was found that the expression of FBN1 was significantly enhanced in cisplatin-resistant OC-like organs and tissues, which may be a key factor in chemotherapy resistance (Wang Z. et al., 2022). Lõhmussaar et al. established an *in vitro* tumor model of fallopian tubes and ovarian surface epithelium (OSE) by targeting the mouse *Trp53* gene alone or in combination with *Brca1*, *Pten*, and *Nf1*. The mouse fallopian tubes and OSE tissues were dissected, and organoids were constructed using the matrix gel method. Additionally, the addition of FGF2 during the initial passage period in the culture medium of the OSE organoids improved the growth of organoids (Lõhmussaar et al., 2020b). By cultivating these two types of organs, it has been proven that both the surface epithelial cells of the fallopian tubes and ovaries can cause high-grade serous OC. Maenhoudt et al. established organoids by using tissue derived from HGSOC patients by the matrix gel method. Furthermore, by testing a variety of media components, it was ultimately determined that the addition of neuromodulin-1 (NRG1) was the optimal key factor to enable the development and growth of OC organoids (Maenhoudt et al., 2020). Cesari et al. collected biopsies from patients, placed them in culture dishes containing AdDF+++medium, chopped them, and digested them in AdDF+++medium containing RHO/ROCK pathway inhibitors to form high-grade serous cancerous organoids from the patient's source through matrix gel method (Cesari et al., 2023). Wang et al. extracted tissues from 20 patients with serous OC, cut them into small pieces, and incubated them in dispersed enzymes. Then, undigested tissue fragments were filtered and removed and embedded in a matrix gel growth factor-reduced basement membrane matrix without phenol red, ultimately forming OC-like organs (Wang W. et al., 2022). Hoffmann et al. obtained specimens from highly purified tumor deposits in the peritoneum or omentum of patients with HGSOC, inoculated in Matrigel matrix gel and supplemented with growth factor cultures to establish omental histologically derived organoids. They found that activation of the Wnt pathway could lead to growth arrest of these organoids and that active BMP signaling was required for the generation of HGSOC organoids (Hoffmann et al., 2020).

In addition to matrix gel method, micro seeding and hydrogel methods are also some of the main methods for serous OC-like organoids. For example, Phan et al. successfully cultured ovarian tumor-derived organoids by using the micro ring seeding method to determine drug sensitivity (Phan et al., 2019). Unlike traditional cultivation methods, by adjusting the geometric shape of tumor cells in the Matrix, single-cell suspensions obtained from cell lines or pre mixed samples with cold matrix gel were plated into a circular shape around the edge of the well plate to generate micro rings around the edge of the well. The results showed that compared with traditional cultivation methods, the micro ring seeding method not only had no impact on growth and drug treatment but could also perform automated high-throughput screening (Phan et al., 2019). Pietilä et al. used the hydrogel method to construct OC-like organs, prepared a hydrogel rich in laminin, and screened the 3D culture cell model from the extracellular matrix (Pietilä et al., 2021). To evaluate the chemical resistance of high-grade serous OC in a tissue-like environment, freshly isolated HGSC cell clusters derived from ascites from patients who underwent one tumor removal surgery or neoadjuvant chemotherapy were treated in COL1 or COL1+COL6 medium for 4 days, followed by 72 h of treatment. After 4 days of 3D culture, these short-term organoids exhibited HGSC morphology, invasive growth in collagen 1 (COL1), and were positive for PAX8 and CK7. COL6 reduced the content of relatively metabolically active cells in 2/4r HGSC-like organs, while in p-HGSC, the activity remained unchanged between matrices or increased + COL in COL1. Notably, among all r-HGSC short-term organoids, COL6 confers cisplatin resistance, while p-HGSC is not affected by treatment and may even become increasingly sensitive. In summary, these results indicate that cisplatin enhances the adhesion of COL6, and COL6 enhances specific protection against cisplatin cytotoxicity in r-HGSC cells. In addition, the protective effect of COL6 can be derived from the intrinsic platinum-resistance mechanism, which is already active in HGSC cells derived from recurrent diseases. It has also been proven that gradually altering the intrinsic adhesion signal transduction and surrounding the extracellular matrix (ECM) of cancer cells to enhance platinum chemotherapy itself can enhance drug resistance. Specific ECM can be achieved through Focal Adhesion Kinase (FAK), and β1 whole protein pMLC-YAP signaling pathway increases resistance to platinum-mediated apoptosis–induced DNA damage (Pietilä et al., 2021).

2.1.2 Other types of OC organoids

For other types of OC models, the matrix gel method is still a commonly used culture method. Zhang et al. obtained ovarian tumor tissues from patients undergoing surgery and established ovarian epithelial cancerous organoids using the matrix gel method. This study observed the morphology of initial organoid cell clusters on a daily basis (Zhang L. et al., 2023). Given the fact that OC organoids originate from different patients, there is significant tumor heterogeneity. The initial OC organoid cell clusters will gradually form OC organoids, which can exhibit cystic or solid growth under light microscopy and gradually increase in volume under 3D culture. By verifying the morphological structure of OC organoids and the expression of molecular markers, the 3D organoid cell viability staining method is used to detect the activity status of OC organoid cells (Zhang L. et al., 2023). Wu et al. obtained OC tissue through biopsy of OC patients and established OC organoids using the matrix gel method. To explore the impact of OC on neutrophils, researchers established a co-culture system of neutrophils and OC-like organs, which is an *in vitro* model that can effectively simulate tumors *in vivo*. By constructing OC-like organs to stimulate neutrophils, the final results showed that MUC16 (CA125) induced neutrophil inflammatory response, thereby promoting the development of systemic excessive inflammation in patients with OC. The factors upregulated by neutrophil inflammatory response can lead to immunosuppressive tumor microenvironment and inhibit NK cells (Wu et al., 2023).

In addition, the simultaneous preparation of various types of OC organoids for comparative research is also a current trend. For example, Kawata et al. prepared OC organoids using matrix gel method, which were prepared from surgical excess or abdominal fluid from OC patients from clinical specimens, including excess abdominal fluid from OC patients during surgery. This study investigated the role of the top basal polarity of OC cell clusters in peritoneal dissemination by utilizing OC organoids from various histological types (Kawata et al., 2022). It was found that polarity switching mediated by SRC family kinases (SFK) is associated with peritoneal metastasis, and polarity switching will be a potential therapeutic target for inhibiting peritoneal dissemination of OC (Kawata et al., 2022). Nanki et al. established different histological subtypes (HGSC, EM, CCC) of organs using the matrix gel method. It was found that organoids cultured with cocktail medium are most effective for multi tissue culture. The overall success rate of organoid culture was 80%, ultimately capturing the histological features and p53 positivity of the primary tumor (Nanki et al., 2020).

2.2 Drug testing and screening

In the past few years, the application of OC organoids in promoting precision medicine, drug detection, and screening is rapidly expanding. Previous studies have mainly focused on gene modification of OC organoids, or constructing organoids using tissues with different gene mutations to construct different OC disease models for drug efficacy evaluation, drug screening experiments, or drug sensitivity testing (Wang W. et al., 2022; Liu et al., 2022; Cao et al., 2023).

2.2.1 Drug efficacy evaluation

Cesari et al. evaluated the drug efficacy of THZ531, a CDK12/13 inhibitor, using organoids and analyzed its effects on HGSOC cells and patient-derived organoids. The results confirmed the strong anti-cancer activity of cyclin-dependent kinase 12 and 13 (CDK12/13) inhibitors and the synergistic effect of THZ531 and pathway inhibitors (EGFR, RPTOR, ATRIP) regulated by cancer-related genes on the activity of HGSOC organoids (Cesari et al., 2023). Wang et al. utilized organoids to investigate Wnt/β-catenin. The efficacy evaluation of the serial protein inhibitor CWP232291 was conducted, and CWP232291 inhibited by β-chain proteins significantly slowed down the growth of OC and could also inhibit the growth of cisplatin-resistant cell lines and organoids derived from patients with serous OC patients (Wang W. et al., 2022).

2.2.2 Drug screening test

The organoid derived from the patient is a suitable *in vitro* model that can be used to screen OC drugs. Zhang et al. used OC-like organs to study the mechanism of acquired resistance to olapanide, a polyADP ribose polymerase inhibitor (PARPi) (Zhang X. et al., 2023). They induced the formation of polyploid giant cancer cells (PGCCs) in ovarian and breast cancer cell lines, organs derived from high-grade serous cancer (HGSC), and patient-derived xenografts (PDX). The results showed that mifepristone blocking the formation of olapanide-induced PGCC could be applied to HGSC-like organ models, and targeting PGC could enhance the therapeutic response to PARPi and overcome PARPi-induced drug resistance (Zhang X. et al., 2023). Cao et al. used OC organoids to study the resistance mechanism and mechanism of action of PARPi, and treated patient-derived organoids with PARPi. PARPi inhibits cell growth by upregulating early cell apoptosis, and PARPi treatment has complex effects on potential gene changes related to PARPi resistance, providing ideas for further research on PARPi resistance mechanisms (Cao et al., 2023). Gray et al. extracted tissue from a patient with platinum-resistant advanced low-grade serous ovarian cancer (LGSOC) who had failed standard chemotherapy and two surgeries. The sample exhibited >70% tumor cell viability, and after 7 days of cultivation using matrix gel method, over 70% pure organoid culture was obtained for drug screening (Gray et al., 2023). After successfully establishing SOC, Vernon et al. identified the broad anti-tumor properties of miR-3622 b-5 p through functional miRNA screening and revealed a new therapeutic combination strategy for ovarian tumor organoids. EGFRi and ABT-737 synergistically act on OC organoids, while the combination of erlotinib and ABT-737 significantly reduces the cell viability of all Patient-derived organoid (Vernon et al., 2020).

2.2.3 Drug sensitivity testing

Ovarian cancer organoids have significant utility in drug sensitivity testing. For example, in the application of SOC organoids, Gorski et al. obtained tissues from OC patients undergoing tumor reduction surgery and developed HGSOC organoids using matrix gel method to test their sensitivity to carboplatin (Gorski et al., 2021). They found that the sensitivity and resistance can be related to the interaction between the NFkB pathway, PRDM6 activation, B-cell receptor signal transduction,

and PI3K-AKT signal transduction pathway (Gorski et al., 2021). Lõhmussaar et al. established HGSOC-like organs from mouse fallopian tubes and ovarian epithelial tissues, discovering the different lineage-dependent sensitivities of common drugs in HGSOC (Lõhmussaar et al., 2020b). In the fallopian tube lineage, after obtaining more mutations, cell lines are usually more sensitive to paclitaxel and niraparib. In the OSE lineage, mutant lines showed lower sensitivity to paclitaxel and niraparib. *In vitro* drug testing using organoids showed that patients have different sensitivities to common drugs used to treat HGSOC (Lõhmussaar et al., 2020b). Maenhoudt et al. used HGSOC organoids for screening of nutlin-3 drugs and found that EOC-derived organoids corresponding organoids (EOC-O-7) organoids derived from p53 wild-type tumors are sensitive to nutlin-3, while EOC-O4 and EOC-O8 organoids established by p53 mutant tumors are insensitive to nutlin-3. The study showed that this organoid can simultaneously perform sensitivity testing of multiple nutlin-3 drugs to different types of tissues (Maenhoudt et al., 2020).

In terms of other types of OC organoids, Kopper et al. successfully established epithelial OC organoids for drug sensitivity testing. They tested the sensitivity of OC organoids to platinum or paclitaxel drugs and found that the HGS-3.1 organoid line is highly sensitive to drugs such as gemcitabine, carboplatin, and paclitaxel, while exhibiting resistance to drugs targeting the PI3K/AKT/mTOR pathway (Kopper et al., 2019). Nanki et al. successfully established OC organoids for drug screening and sensitivity testing, including advanced serous, clear cell, and endometrioid cancers. They found that organoids carrying the pathogenic variant of BRCA1 were more sensitive to PARP inhibitor olapanib and platinum-based drugs, while organoids from clear cell OC were resistant to conventional drugs such as OC platinum-based drugs, paclitaxel, and olapanib (Nanki et al., 2020).

2.3 Patient stratification and drug response prediction

The inherent molecular heterogeneity is particularly prominent in OC, which leads to differences in drug responses among different patients (Yang et al., 2021). Organ-like models that can preserve the heterogeneity and genetic characteristics of the original tumor have advantages in precision medicine. Organ-like models taken from different patient tissues have different drug responses, and stratifying individual patients into customized plans has broad prospects.

2.3.1 Patient stratification

Sauriol et al. evaluated the response of three high-grade SOC organoids, with one patient resistant to platinum and two patients sensitive to platinum; the latter two received maintenance treatment with olaparib but relapsed, indicating their resistance to olaparib. *In vitro* experiments showed that two models were sensitive to olapanib and one was resistant. The combination of olaparib and nicotinamide phosphoribosyltransferase (NAMPT) inhibitors has a synergistic effect and effectiveness on all three models, including clinically acquired PARPi-resistant models. This study combines PARPi with NAMPT inhibitors and applies them to a PARPi-resistant HGSOC organoid model. The results show that intracellular NAD+is depleted, inducing double stranded DNA breakage and promoting cell apoptosis through caspase-3 cleavage monitoring. In the context of PARPi resistance, NAMPT inhibition provides a promising new option for OC patients (Sauriol et al., 2023).

Gray et al. reported that a platinum-resistant advanced low-grade SOC patient who failed standard chemotherapy and two surgeries rapidly deteriorated into end-of-life care, and genomic analysis of the tumor did not indicate a clear treatment option either. After determining several treatment options through drug sensitivity testing of the patient's tumor-like organs, the patient achieved remarkable clinical transformation with subsequent treatments, highlighting the clinical efficacy of *in vitro* drug testing of tumor-like organs from the patient's source as a new functional precision medicine method, which can determine effective personalized therapies for patients who have failed standard care treatments (Gray et al., 2023).

Tao et al. found that among three epithelial OC organoids with homologous recombination repair (HRR) defect mutations, two were sensitive to PARPi and one was congenitally drug-resistant. Another type of organoid derived from relapsed patients during olaparib maintenance therapy was found to develop acquired resistance to PARPi. The subsequent functional analysis revealed potential drug resistance mechanisms associated with replication cross protection and HRR functional recovery. Combination strategies targeting these mechanisms can reverse drug resistance, demonstrating the sensitivity of EOC PDO to PARPi in evaluating different environments (Tao et al., 2022).

2.3.2 Drug reaction prediction

Chen et al. obtained tumor specimens in the form of multicellular spheroids (MCS) from malignant exudates of OC patients and formed organoids through primary culture. It was found that the sensitivity of organoids from different samples of the same patient to carboplatin and paclitaxel chemotherapy drugs varies, and drug sensitivity testing can be conducted through organoids (Chen et al., 2020). After constructing epithelial OC organoids, Zhang et al. added different concentrations of carboplatin and calculated the IC50 of carboplatin to OC organoids. The results showed that organoids could be stably passaged *in vitro*, and patients receiving neoadjuvant chemotherapy had higher resistance to carboplatin (Zhang L. et al., 2023). Bose et al. constructed ascitic organoids derived from advanced and/or recurrent high-grade SOC patients and transfected HyPer DAAO to express them. The study found that the HyPer signal in PDOs of carboplatin-resistant patients was significantly higher than that of carboplatin-sensitive patients (Bose et al., 2022). Witte et al. found that organoids can maintain the genomic characteristics of the original tumor lesion, reflecting the patient's response to neoadjuvant carboplatin or paclitaxel combination therapy. PDO shows heterogeneity in drug response to chemotherapy and targeted drugs between and within patients. Using patient-derived organoids for *in vitro* drug screening, 88% of patients were found to have high reactivity to at least one drug through drug screening (de Witte et al., 2020). Sun et al. established organoids from cisplatin sensitive and resistant OC tissue samples and found that serine/threonine kinase (Aurora-A) induces cisplatin resistance by regulating cell aging and glucose

metabolism through involvement in SOX 8/FOXK 1 signaling in OC (Sun et al., 2020).

Ito et al. performed sensitivity testing on paclitaxel and carboplatin using organoids prepared from cancer tissue primary spheroids (CTOS) of OC patients undergoing chemotherapy, with a success rate of 84% (Ito et al., 2023). Extensive sensitivity was observed in organoids of both drugs. Of the 18 patients in whom clinical response information was available, all four clinically resistant organoids showed resistance to both drugs. Among the 18 cases, five had dual drug resistance, and their response rate was consistent with the clinical response rate. It was also found that several drugs combined with carboplatin had better effects than paclitaxel, and some drugs such as afatinib showed cumulative effects with carboplatin (Ito et al., 2023).

Taken together, the above examples indicate that OC organoids derived from patients have a clinical translational role in predicting drug response and resistance mechanisms and play a major role in precision medicine.

3 Endometrial cancer (EC) organoids

The incidence rate and mortality of EC in developed countries have increased significantly every year (Sung et al., 2021). The treatment options for women with advanced EC are limited, and the currently available treatment methods are not ideal. Developing effective and precise targeted therapies requires a real preclinical model, and the organoid model of EC is of great significance for the pathological status of EC.

3.1 Disease modeling

At present, there is no mention of specific histological types for modeling EC organoids. However, by obtaining organoids from patient tissues and constructing EC organoid models, it is helpful for the research of EC. The following experiments and studies show the modeling method for EC organoids in the study.

The matrix gel method is the main culture method for EC-like organs, with the main difference being the tissue source. Directly obtaining tissue samples from EC tissue and then culturing them into EC-like organs is a common method. For example, the EC tissue collected by Xue et al. was subjected to *in vitro* treatment and culture after excision, and organoids were cultured using the matrix gel method (Yang et al., 2023). The specific method is to embed a single cell or organ-like fragment into the ECM of Engelbreth-Holm-Swarm (EHS) mouse sarcoma and distribute it to the surface of the plastic container for warm tissue culture in the form of small droplets. After the ECM is incubated at 37°C, it will solidify into a gel, which can then be covered with the culture medium (Yang et al., 2023). Organoid will develop into 3D structure in the dome. Jamaluddin et al. constructed organoids from tissue biopsies collected from four patients with EC and cultured them using the matrix gel method. They found that proteomic differences observed in the same tumor in patients were also transformed into differences in tumor cell growth rate (Jamaluddin et al., 2022). Su et al. cultured fresh tumor tissues from patients with EC using the matrix gel method and analyzed these organoids with different estrogen-related receptors α (ERR α) Sensitivity of organoids to DDP at expression levels. Studies have shown that the proportion of organoid fragments significantly increases in a dose-dependent manner after cisplatin treatment, with estrogen related receptors α (ERR α) The inhibition promotes the activation of NLRP3/caspase1/GSDMD pyroptosis pathway in EC cells (Su et al., 2023). Berg et al. obtained fresh tumor tissue from patients with malignant endometrial diseases and established EC organoids by using the matrix gel method. They found that removing N2 supplementation and adding ROCK inhibitors can enable long-term expansion and cryopreservation of organoids, which can reflect the genetic characteristics of endometrial tumors and predict patient prognosis through organoid models (Berg et al., 2021).

Some studies have explored new sources of organization and cultivation methods. For example, Katcher et al. collected endometrial samples from patients and used the matrix gel method to establish cancerous and normal endometrial tissue organoids for further analysis for DNA and RNA extraction and histological analysis (Katcher et al., 2023). Hsin et al. used xenografts (PDX) from patients with EC to construct organoids. They subcutaneously implanted tumor samples from EC patients into late stage severely immunodeficient mice, killed the mice, and conducted organoid formation experiments and primary cell culture via the matrix gel method (Hsin et al., 2023). Their study used repeated up and down pipetting to dissolve organoids for passage (Hsin et al., 2023). Sengal et al. collected PDX tumors from EC patients, dissected, and placed some of the tumors in RPMI culture medium containing antibiotics (penicillin/streptomycin) and antifungal drugs, and constructed EC organoids using the matrix gel method (Sengal et al., 2023). Maru et al. used a matrix gel bilayer organoid culture scheme to construct organoids to explore whether the combination of Kirsten rat sarcoma viral oncogene homolog (*Kras*) gene activation and *Pten* inactivation can transform mouse endometrial organoids in the subcutaneous tissue of immunodeficient mice. This study found that in endometrial organoids expressing *Kras* (G12D), *Pten* knockdown does not confer tumorigenicity, but *Cdkn2A* knockdown or *Trp53* deficiency leads to the development of carcinosarcoma (CS). The carcinogenic potential of *Kras* (G12D) and the histological characteristics of derived tumors depend on the environment and vary depending on organ type and the experimental environment (Maru et al., 2021a).

In summary, the main method for modeling EC-like organs is to isolate tumor cells from EC tissue (from surgery, biopsy, xenografts from EC patients, and EC mice); use matrix gel method; chop; rinse; digest; and filter; and embed them into a 3D matrix, and cultivate them in a culture medium supplemented with various growth factors and hormones. However, the specific experimental conditions reported in each study are different and have been specifically listed above. Wu et al. optimized the current EC like organs by introducing cancer-associated fibroblasts (CAFs) isolated from EC lesions (Wu et al., 2022). Based on co-cultivation of CAFs and EC-like organs, they found that CAFs can promote the growth of EC-like organs, possibly by secreting factors. According to the results, CAFs can also promote growth, providing a more promising model for the basic and preclinical research of EC (Wu et al., 2022).

3.2 Drug efficacy evaluation and drug response prediction

Similar to OC organoids, to better simulate the treatment response of tumors, existing studies have constructed EC organoid models, retaining the histological and genetic characteristics of the original tumor as well as tumor heterogeneity to evaluate drug efficacy and predict drug response.

3.2.1 Drug efficacy evaluation

Xue et al. evaluated the effectiveness of the small molecule inhibitor SMIP34, which inhibits PELP1 oncogenic signaling, in the treatment of EC. SMIP34 was used to treat *in vitro* patient-derived explant-like organs and cells, and it was found to significantly reduce cell viability, colony forming ability, and the ability to induce apoptosis (Yang et al., 2023). Spencer et al. evaluated the drug efficacy of LIFR inhibitor EC359 by constructing EC organoids. The organoid viability analysis established through primary type II EC tissue showed that compared with dose-dependent vector therapy, the new small molecule LIFR inhibitor EC359 treatment reduced its viability and inhibited the growth of EC organoids, indicating that LIFR inhibitor EC359 may be a new small molecule therapy for the treatment of type II EC (Spencer et al., 2023). Hsin et al. validated using organoids and primary cells derived from xenografts (PDX) from EC patients β-catenin inhibitory ability of the catenin inhibitor ICG-001 on EC was studied, and the results showed that ICG-001 can inhibit PDX-derived organoids and primary cells (Hsin et al., 2023).

By constructing an EC organoid model to test the drug efficacy of antisense oligonucleotides (ASO), a study found that ASO targeting SNORD14E inhibited the growth of EC (Chen et al., 2023). Chen et al. cultured the mouse EC model with *Trp53*, *Pten*, and *Pik3r1* mutations and overexpression of *Myc* and *Kras* (G12D) in the medium containing matrix gel, established EC-like organs by matrix gel method, screened therapeutic drugs for EC, and found that the menin-MLL inhibitor affects the progress of EC by regulating the HIF pathway. MI-136 significantly inhibits the growth of EC-like organs from patients. The results showed that MI-136 can serve as a potential inhibitor of EC by regulating the HIF pathway (Chen et al., 2021).

3.2.2 Drug reaction prediction

Sengal et al. tested the drug sensitivity of Fibroclast Growth Factor Receiver (FGFR) through the establishment of EC organoids, indicating that PDX-derived organoids and PDX with FGFR 2c subtype expression are sensitive to FGFR inhibition (Sengal et al., 2023). Berg et al. cultured the tumor tissue of excised EC patients into organoids and amplified it in a culture medium determined by chemical composition to predict patient prognosis and provide more effective medication. The OEC-07-G3 cell line was found to be highly sensitive to carboplatin–paclitaxel, and the survival rate measured after combination treatment with carboplatin (200 μM) paclitaxel (200 nM) was only 5.4% (Berg et al., 2021).

All the above examples indicate that EC-like organs derived from patients have a clinical translational role in predicting drug response and resistance mechanisms and play a major role in precision medicine.

4 Cervical cancer (CC) organoids

Cervical cancer is the fourth-most commonly diagnosed and lethal cancer among women (Sung et al., 2021; Zhang et al., 2021). The vaccination rate and cervical screening rate of human papillomavirus (HPV) in low- and middle-income countries are still quite low (Doherty et al., 2016; Spayne and Hesketh, 2021). For decades, this has exacerbated the ongoing burden of CC in developing countries. The treatment options for CC, including surgical resection, radiotherapy, and chemotherapy, have limited efficacy and may have significant toxicity to patients with recurrence or metastasis (Chung et al., 2019). In recent years, immunotherapy has become a promising method for treating CC (Hu and Ma, 2018). However, due to the complexity and heterogeneity of solid tumors, the efficacy of immunotherapy varies among patients (Hegde and Chen, 2020; Ando et al., 2021). Therefore, it is necessary to develop preclinical models that can accurately evaluate the efficacy and mechanisms of these therapies.

4.1 Modeling of CC organoids

At present, cervical small-cell neuroendocrine carcinoma (SCNEC) organoids (Masuda et al., 2023), cervical small-cell carcinoma (SCCC) organoids (Kusakabe et al., 2023), cervical clear-cell carcinoma (cCCC) organoids (Maru et al., 2019a), and squamous cell carcinoma and adenocarcinoma (AdCA) organoids (Lõhmussaar et al., 2021) have been established. By constructing an organoid model of CC in patients, preserving the genomic map of the primary tumor, and identifying the genetic correlations related to the progression of CC can contribute to the study of disease etiology. The lack of a human derived *in vitro* model that can summarize cervical precancerous lesions has always been a bottleneck in the study of HPV infection-related precancerous lesions and cancer. By constructing an organoid model covering patient sources of HPV-related cervical precancerous lesions and cancer, preserving genomic and transcriptomic features, as well as pathogenic HPV genome, an experimental platform and biological library have been established for the *in vitro* mechanism research, therapeutic vaccine screening, and personalized treatment of HPV-related cervical diseases (Kusakabe et al., 2023; Hu et al., 2024).

Masuda et al. used the matrix gel method to prepare SCNEC organoids from patient tumors or mouse xenografts. Histologically, organoids and xenograft tumors showed clear differentiation into SCNEC or AdCA in certain areas and unclear differentiation in some areas (Masuda et al., 2023). By tracking single cells, the existence of cells with dual potential differentiation towards SCNEC and AdCA was revealed. Single-cell transcriptome analysis identified three distinct clusters: SCNEC-like clusters, AdCA-like clusters, and clusters lacking specific differentiation markers. The expression of neuroendocrine markers is enriched in SCNEC-like clusters, but not completely enriched. HPV 18 E6 is enriched in SCNEC-like clusters, exhibiting higher proliferation and lower levels of p53 pathway. After anti-cancer drug treatment, the expression of AdCA markers is increased, whereas the expression of SCNEC is decreased. The report system using keratin 19 expression revealed that the changes in cell differentiation were related to

the differentiation transformation induced by drug therapy. These data indicate that mixed SCNEC/cervical tumors have a clonal origin, characterized by unclear differentiation status (Masuda et al., 2023).

Kusakabe et al. obtained surgical specimens from a patient diagnosed with HPV18-positive SCCC and performed organoid culture using the matrix gel method (Kusakabe et al., 2023). The results of HPV18-positive SCCC organoids culture and drug sensitivity tests using mouse xenograft models derived from the organoids showed that KRAS pathway inhibitors had stronger anti-cancer effects on SCCC organoids than the Myc inhibitors, which was also confirmed in the xenograft models (Kusakabe et al., 2023).

Maru et al. successfully established the first cCCC organoid using the matrix gel method, demonstrating the sensitivity of cCCC to major chemotherapy drugs and MET inhibitors. This indicates that the organoid derived from the tumor retains the morphology and genetic abnormalities of the original tumor, providing a reference for the treatment of cCCC (Maru et al., 2019a). Lõhmussaar et al. collected materials from Pap smears of patients with squamous cell carcinoma (SCCa) and AdCa CC and established CC-like organs using the matrix gel method to study cervical histological dynamics. After successfully establishing a 3D CC-like organ, personalized medical methods for CC were studied (Lõhmussaar et al., 2021). Hu et al. established a long-term 3D organoid culture protocol using the matrix gel method, and established an organoid model covering patient sources of HPV-related cervical precancerous lesions and their cancers. The model retained genomic and transcriptomic characteristics as well as pathogenic HPV genome. This study was the first to establish a cervical precancerous lesion model containing HPV, providing an experimental platform and biological library for *in vitro* mechanism research, therapeutic vaccine screening, and personalized treatment of HPV-related cervical diseases (Hu et al., 2024).

Toyohara et al. constructed CC organoids using the matrix gel method, transfected the lentiviral vector (HPV18LCR-GFP vector) into squamous columnar junction (SCJ) organoids from patients, and evaluated the presence of green fluorescence protein (GFP)-positive cells (Toyohara et al., 2023). The results showed that pathways related to cell cycle and viral carcinogenesis were upregulated in GFP-positive cells, while keratinization and mitochondrial autophagy/autophagy-related pathways were upregulated in GFP-negative cells. Among the upregulated genes, *ADNP*, *FHL2*, and *NPM3* were significantly associated with the activation of the early promoter of HPV18 and the maintenance of the HPV18 gene group. Thus, the initial replication mechanism of HPV18 and the breakthrough in the origin of HPV18-related CC cells were determined (Toyohara et al., 2023).

In summary, the main method for modeling CC organoids is by constructing organoids using the matrix gel method by isolating tumor cells from CC tissue (obtained by surgery, biopsy, smear, patient tumor, or mouse xenograft). The key to successful organoid culture is digestion of the original tissue and composition of the culture system. During the digestion process of the original tissue, it is necessary to increase the number of cells as much as possible while ensuring cell viability. Therefore, Hu et al. constructed an organoid model covering patient sources of HPV-related cervical precancerous lesions and their cancers, and innovatively used an enzyme mixture called SIL tissue dissociation solution to obtain a sufficient number of cells to the maximum extent possible (Hu et al., 2024). In addition, the tissue obtained from constructing organoids varies. Toyohara et al. constructed organoids by obtaining normal tissue from the squamous columnar junction area of patients undergoing a total hysterectomy. The specific experimental conditions reported in each study of organoids vary, as detailed in the above sections (Toyohara et al., 2023).

4.2 Drug testing and screening

The application of organoids in promoting precision medicine, drug detection, and screening is rapidly expanding (Kopper et al., 2019; Shi et al., 2020). To better simulate the treatment response of tumors, organoids were constructed using tissues from different patient sources, and different CC disease models were constructed. The histological and genetic characteristics of the original tumor as well as tumor heterogeneity were preserved, and drug efficacy evaluation or drug sensitivity testing and drug screening experiments were carried out (Lin et al., 2023a; Hu et al., 2023).

Hu et al. explored the novelty of the therapeutic effect of synchronous radiotherapy and chemotherapy on CESC by constructing organoids for the same (Hu et al., 2023). The research results showed that high expression of Lumican (LUM) affected the immune microenvironment of organoids in patients with CESC treated with synchronous radiotherapy and chemotherapy. High expression of LUM was associated with poor efficacy in CESC patients receiving synchronous radiotherapy and chemotherapy, possibly by affecting the PAR and IL1 signaling pathways of the immune landscape (Hu et al., 2023). Lin et al. explored the sensitivity of drug action by constructing CC-derived organoids and found a significant heterogeneity in carcinogenic and tumor microenvironment between CSCC and CAde pathological types. CAde has a more inhibitory immune microenvironment, and lapatinib (an ERBB2 inhibitor) is particularly sensitive to CAde samples. Dasatinib and Doramamod targeting STAT5 and MAPK molecules exhibit specific sensitivity to CSCC cancer cell lines and organoids (Lin et al., 2023a).

Fonte et al. explored the therapeutic effect of the combination of trabectedin and propranolol by constructing organoids. Research has shown that trabectedin can reduce the proliferation of organoid cell lines derived from CC patients, and trabectedin is associated with β-blocker propranolol combined therapy to counteract the effects in CC models β- Activation of adrenergic receptors leads to resistance to trabectedin (Di Fonte et al., 2023). Lõhmussaar et al. established CC organoids in SqCa and AdCa and evaluated possible p53 pathway defects in organoids using the p53-activating compound, Nutlin-3a (Lõhmussaar et al., 2021). According to genomic data, the p53 mutant SqCa-1.2 line is the most resistant, and the SqCa-3 line shows the highest resistance to treatment with two platinum analogues, while the SqCa-6 and SqCa-7 lines are highly sensitive to gemcitabine (Lõhmussaar et al., 2021). The PDO biobank established by Huang et al. contains 67 cases of heterogeneous CC organoids, and their *in vitro* responses indicate that they can capture the radiation heterogeneity of patients (Huang et al., 2023). To simulate an individual's response to adoptive T cell therapy (ACT), tumor infiltrating

lymphocytes (TILs) were amplified *in vitro* and co-cultured with paired organoids. The PDOs TILs co-culture system showed significant response, supporting the potential of the PDOs platform in guiding prospective intervention trials for CC treatment (Huang et al., 2023).

All the above examples indicate that CC organoids derived from patients have a clinical translational role in drug screening and predicting drug reactions and resistance mechanisms and play a major role in precision medicine.

We have summarized the research progress of OC, EC, and CC organoids in Figure 1.

5 Discussion

5.1 Organoids and the three major gynaecological malignant tumors

5.1.1 Main modeling methods, application fields, and future directions of OC organoids

In terms of disease modeling, various histological subtypes of organoids have been successfully developed, including SOC organoids, clear cell carcinoma, endometrioid OC, and epithelial OC organoids. Based on the above summary of modeling methods for OC organoids, we found that the main method for modeling OC organoids is to construct organoids using the matrix gel method by isolating tumor cells from OC tissue (from surgery, biopsy, ascites or pleural effusion, high-purity tumor deposits in the peritoneum/omentum). However, the specific experimental conditions reported in each study vary; for example, some studies have adopted improved methods by adding advanced DMEM/F12 medium containing specific growth factors and niacinamide (Willert et al., 2003). In addition, some studies have used the hydrogel method to construct OC-like organs (Pietilä et al., 2021). Phan et al. found that this cultivation method not only did not affect the growth of organoids but also afforded automated high-throughput screening of chemotherapy drugs when using the micro ring seeding method (Phan et al., 2019). When multiple tissue types of organoids are cultivated simultaneously, using a cocktail medium to cultivate organoids can achieve a total success rate of 80% (Nanki et al., 2020). In addition, the use of Accumax 7-min enzymatic hydrolysis increased the success rate of organoid proliferation in tumors of different stages and subtypes from 45% to 90% (Maru et al., 2019b). In terms of medium additives, Hoffmann and Lõhmussaar et al. found that epidermal growth factor (EGF) is an essential component, and the addition of neuromodulatory protein-1 (NRG1) is the optimal key factor for the development and growth of OC-like organs (Lõhmussaar et al., 2020b; Hoffmann et al., 2020; Maenhoudt et al., 2020). In addition, in the extraction of OC organoids, establishing organoids derived from omental histology facilitates the exploration of relevant metabolic mechanisms (Lõhmussaar et al., 2020b). By constructing a model of organoid diseases and constructing OC models with different gene expressions, we aimed to explore the relationship between genes and the occurrence, development, and prognosis of OC (Iwahashi et al., 2022; Raab et al., 2024); identify ligands and receptors associated with poor survival in OC (Carvalho et al., 2022); study chemotherapy resistance mechanisms (Gorski et al., 2021); explore platinum-based chemotherapy response and survival markers for OC (Wang Z. et al., 2022; Compadre et al., 2023); and explore the role of signaling pathways in OC (Hoffmann et al., 2020). In terms of drug detection and screening, by constructing an OC organoid model and summarizing the characteristics of the tumor, drug screening experiments (Nanki et al., 2020; Vernon et al., 2020; Wang W. et al., 2022; Zhang et al., 2022a, 2022b; Liu et al., 2022; Cesari et al., 2023; Wan et al., 2023) and drug sensitivity tests (Kopper et al., 2019; Lõhmussaar et al., 2020b; Maenhoudt et al., 2020) can be conducted to explore the mechanisms of drug

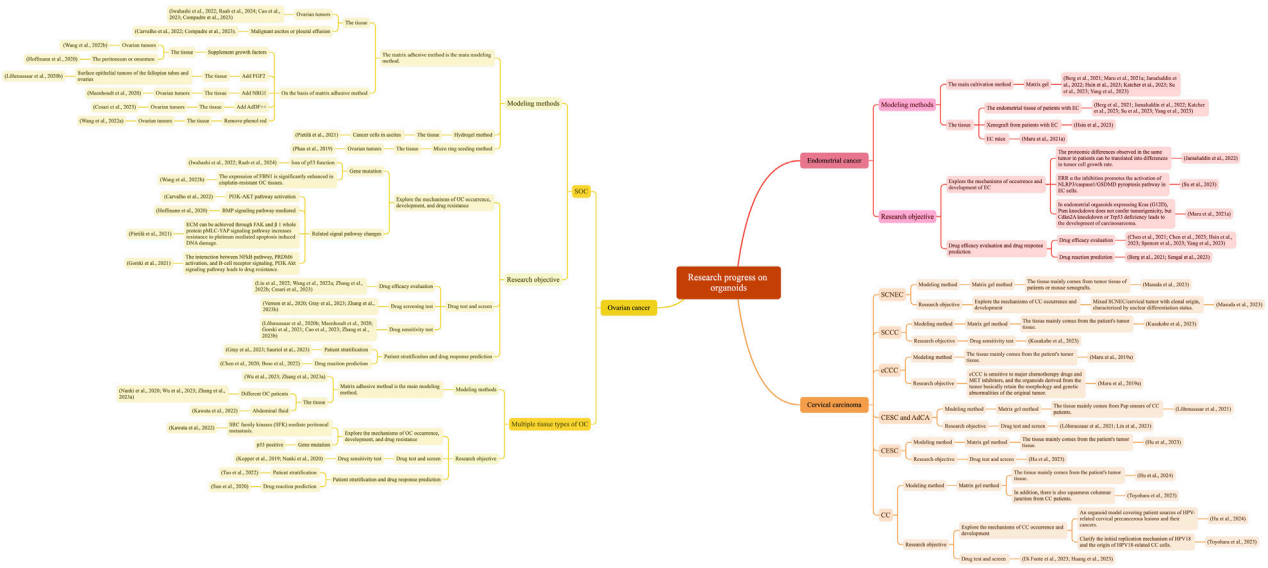

FIGURE 1
Research progress on organoids of OC, EC, and CC with Xmind.

resistance and drug action in OC (Zhang X. et al., 2023; Cao et al., 2023). Meanwhile, molecular heterogeneity exists in OC, leading to differences in drug responses among different patients. Organs that retain the heterogeneity and genetic characteristics of the original tumor have an advantage in precision medicine. *In vitro* drug testing of OC organoids from patient sources is a new method in precision medicine (Chen et al., 2020; Zhang L. et al., 2023; Gray et al., 2023; Sauriol et al., 2023).

5.1.2 Main modeling methods, application fields, and future directions of EC organoids

In terms of disease modeling, the currently developed EC organoid modeling can be stored for a long time, and can be used for downstream histological and genomic characterization as well as functional determination, such as evaluating response to therapeutic drugs (Katcher et al., 2023). Moreover, the proteomic differences in organoid tissues are consistent with the growth rate of tumor cells (Jamaluddin et al., 2022). Therefore, organoids that can highly preserve the structure and function of primary tissues have broad prospects in exploring the mechanisms driving tumor development (Maru et al., 2021a; Sahoo et al., 2022). At present, the main method for modeling EC organoids is to isolate tumor cells from EC tissue and use the matrix gel method to construct organoids. However, different specific methods have not been studied. Most studies focus on EC patients, but some studies have constructed organoids by collecting EC mouse tissue (Sahoo et al., 2022), and some others have transformed mouse endometrial organoids into subcutaneous tissue (Maru et al., 2021a). Another study has found that removing N2 from the culture medium and adding ROCK inhibitors can promote long-term expansion and cryopreservation of organoids (Berg et al., 2021). Wu et al. optimized the current EC-like organs by introducing CAFs isolated from EC lesions (Wu et al., 2022). Based on co-cultivation of CAFs and EC-like organs, they found that CAFs can promote the growth of EC-like organs, possibly by secreting factors. According to the results, CAFs can also promote growth, providing a more promising model for the basic and preclinical research of EC (Wu et al., 2022). In addition, EC organoids can be used for drug detection and screening (Chen et al., 2023; Hsin et al., 2023; Spencer et al., 2023; Su et al., 2023; Yang et al., 2023) and drug screening (Chen et al., 2021) to explore the efficacy and mechanism of drug action. In addition, by constructing organoids from patient sources, it is possible to accurately predict the effects of drug action on different patients, playing a role in precision medicine (Berg et al., 2021; Sengal et al., 2023).

5.1.3 Main modeling methods, application fields, and future directions of CC organoids

Published studies have reported the establishment of SCNEC organoids, SCCC organoids, cervical transparent cell carcinoma organoids, CESC organoids, and AdCA organoids, constructed CC organoids, captured the characteristics of histological cancer subtypes, and constructed organoid models covering patient sources of HPV-related cervical precancerous lesions and their cancers. Studies have also established the experimental platforms and biobanks for *in vitro* mechanism research, therapeutic vaccine screening, and personalized treatment of HPV-related cervical diseases (Kusakabe et al., 2023; Hu et al., 2024). At present, the main method for modeling CC organoids is to isolate tumor cells from CC tissue. In addition, some studies have also constructed organoids using the matrix gel method by using normal squamous columnar junction tissue from patients undergoing total hysterectomy (Toyohara et al., 2023). In addition, studies have shown that culture media play an important role in constructing organoids of different tissue types (Huang et al., 2023). The most important key to successful organoid culture is the digestion of the original tissue and the composition of the culture system. During the digestion process of the original tissue, it is necessary to increase the number of cells as much as possible while ensuring cell viability. Therefore, studies have also attempted to improve the treatment of CC tissue with tissue dissociation solution to obtain a sufficient number of cells to the maximum extent (Hu et al., 2024). Moreover, CC organoids can also be used for the detection of different drugs (Di Fonte et al., 2023; Hu et al., 2023) and sensitivity testing (Lin et al., 2023a). Similar to OC and EC, CC organoids that highly preserve the heterogeneity of the original tissue can be used for personalized and precise treatment, exploring drugs for experimental treatment in patients with different gene mutations (Lõhmussaar et al., 2021; Huang et al., 2023).

5.2 Organoid modeling application insights

5.2.1 Limitations of existing tumor models

Gynecological malignant tumors are major diseases that threaten women's lives, and traditional treatments fail to ensure the efficient killing of tumor cells by drugs and a good prognosis after treatment, mainly because the tumor and its microenvironment are not well understood. Therefore, it is crucial to find a suitable model that is stable and representative of the complex structure of human tumor tissues and other tissues. Popular tumor models in recent years mainly include traditional cell line models and xenograft models. However, these models have limitations, perhaps not replicating well the tissue complexity and genetic heterogeneity of tumors, poorly reproducing the clinical response of patients, and failing to predict clinical response.

Traditional cell line models mainly refer to 2D cell line models. 2D cell cultures lack the ability to regulate cell behavior, cannot be modeled based on cell-cell/cell-ECM interactions, and often adhere to the surface of the plastic medium to form a monolayer of cells, which are directly and uniformly exposed to intra-medium factors that fail to replicate the actual microenvironment of the tissues, whilst the 2D *in vitro* culture conditions require extensive selection and adaptation, and since only rare clones are able to expand and maintain multiple passages, derived cell lines may have undergone substantial genetic changes that make it difficult to reproduce the genetic heterogeneity of the original tumor (Li and Zhang, 2023; Cheng et al., 2024). Peng D. et al. developed CancerCellNet technology based on transcriptomic analysis for assessing the match between different cancer models and primary tumors *in vivo*, and showed that human cancer cell lines are not genetically identical to cancer cells *in vivo*, suggesting that highly transmissible cell lines sometimes undergo potential evolution, and that the 2D cell line models do not accurately represent the genotypic and pathological characteristics of the primary tumor cells (Peng et al., 2021).

Xenograft models are able to mimic human tumor biology, although the limitations are limited efficiency and the high cost of transplantation for subpopulations of patient tumors (Li and Zhang, 2023). Yuan Z. et al. analyzed unlocalized RNA-Seq reads from 184 experiments to assess the extent of viral infection and its effect on patient-derived xenografts, and found that a certain anticancer drug turned out to appear to kill tumor cells, when in fact, because human tumors are infected by mouse viruses when implanted in mice, many anti-cancer drugs that kill tumor cells in mouse models do not work in human trials (Yuan et al., 2021).

5.2.2 Characteristics of organoid modeling applications

Organ-like structures are simplified or formed in 3D culture systems and can reproduce the structure and physiology of most female reproductive tissues (Kessler et al., 2015; Turco et al., 2017). Experiments on organs provide a unique opportunity, as they are translatable, repeatable, and scalable. They are produced from pluripotent stem cells or adult stem cells, making them a special 3D culture system that can closely mimic the structure and physiology of the originating tissue (Kim et al., 2020). Given the high heterogeneity of gynecological tumors, organoids can highly reproduce the original characteristics of tumors *in vivo* and *in vitro*, and have broad application potential in disease mechanism research, drug research, predicting patient response to treatment, and providing personalized medical solutions for patients (Liu et al., 2020). Gynecological cancer organoids are widely used as the best model for studying tumor biology (Salinas-Vera et al., 2022). This is because using this model system enables scientists to better understand the development mechanism of tumors, disease progression, and drug efficacy, thereby providing patients with more reliable treatment options. These studies are of great significance for discovering new anti-cancer drugs and improving patient survival rates. Overall, the application of gynecological cancer organoids in the field of tumor biology has played a crucial role in the development of clinical medicine. Moreover, gynecological tumors are more complex, and different patients may have significant differences in their response to the same clinical treatment. For gynecological tumors with extremely high malignancy, the mechanisms of tumor progression, drug efficacy, and drug resistance remain unclear (Tendulkar and Dodamani, 2021). A large number of anti-cancer drugs that have passed Phase-I drug safety tests have been eliminated in Phase-II and III efficacy tests, while patient-derived tumor organoids (PDTO) can optimize preclinical efficacy in models to improve the prediction of clinical treatment responses and reduce the failure rate of clinical trials, thus solving this important problem (Jin et al., 2020). Gynecological patients often exhibit resistance to chemotherapy and radiation therapy, and traditional treatment methods may not be effective in inhibiting tumor growth and metastasis. Some patients may even receive overly toxic treatments without significant results (Kopper et al., 2019). By using organoid culture models, researchers can predict the patient's response to different treatment methods, thereby tailoring specific treatment plans for each patient. Through personalized treatment, doctors can more accurately select drugs and doses that are suitable for patients and adjust them according to the patient's condition at any time. In this way, patients can achieve better treatment outcomes without having to endure the side effects of excessively toxic drugs, thereby achieving precise treatment. In addition, most gynecological tumors progress rapidly, and patients are often diagnosed in the middle and late stages. Hence, it is particularly important to develop a suitable treatment plan. We can predict the response of patients to biopsy tissue treatment through organoids to select the most suitable treatment plan for patients (Lõhmussaar et al., 2020a).

We should acknowledge the limitations associated with organoid-based treatments, such as a lack of scalable organoid lineages with the ECM and immune components, and a lack of interaction with native ECM components (Francés-Herrero et al., 2022). Organs exhibit relatively random growth properties, do not support interorgan communication, and lack vascular systems and immune cells (Shankaran et al., 2021). The random distribution of organoids in the matrix gel can cause differences in spatial density and spacing, leading to uncontrolled variations in organoid phenotype. The variability related to patient tissue origin and culture treatment still needs to be further optimized. In addition, the differences in matrix gel (including differences in physical properties and growth factor content) can directly affect the stability of organoid cultures, and frequent manual operations during the cultivation process can introduce human errors, thereby affecting the stability of the culture system. Therefore, to improve the success rate of organoid generation and research, it is crucial to develop standardized protocols for routine organoid-based treatment (Clinton and McWilliams-Koeppen, 2019). The current results show that the synthetic guiding hydrogel can closely imitate the structure and characteristics of the natural ECM in cancer tissue and has appropriate clues to support tumor growth, migration and organoid invasion *in vitro*, so it has a potential in future organoid culture research (Jia et al., 2022).

5.2.3 Prospects for the application of organoid modeling

5.2.3.1 Range of diseases

In summary, we found that these three types of organs have gradually developed to the stage of technological maturity through a review of OC-like organs, EC-like organs, and CC-like organs. At the same time, more types of gynecological tumor-like organs are also being developed. Lõhmussaar et al. established an *in vitro* model of fallopian tube and ovarian surface epithelial cell (OSE) tumor development by targeting the mouse *Trp53* gene (Lõhmussaar et al., 2020b). Mouse fallopian tubes and OSE tissues were dissected, subjected to different enzyme treatments, embedded in basement membrane extract (BME), and cultured in appropriate media to ultimately cultivate fallopian tube- and ovarian tumor-like organs (Lõhmussaar et al., 2020b). Subsequently, Maru et al. utilized mouse fallopian tube organoids to investigate the tumorigenic potential of recombinant gene interactions (Maru et al., 2021b). The results showed that inhibition of *Pten* and simultaneous induction of *Pik3ca* mutation led to the development of *in situ* cancer and high-grade serous tumors, respectively, reflecting the frequent activation of the PI3K/AKT axis and the impact of activation of the Wnt pathway in HGSC on tumor generation (Maru et al., 2021b). Meanwhile, studies have also established a mouse model of fallopian tube organoids and found that the ALDH1A family inhibitor (ALDHi) 673A reduced organoid

complexity and significantly reduced colony formation in *BRCA* mutant cells, suggesting that ALDHi 673A can serve as a chemopreventive agent for *BRCA1/2* mutation carriers (McGonigal et al., 2023). Yang et al. established cancerous organoids in the ascites of OC patients and found that miR-1246 and miR-1290 shuttle through malignant ascites-derived extracellular vesicle (EVs) by regulating the common target ROR α to promote the invasion and migration of OC cells (Yang et al., 2022). Recently, Diaz et al. generated patient-derived organoids from liquid biopsies of patients with gynecological serous carcinoma (GSC) and found that the histological and immunofluorescent characteristics of ascites-derived organoids were similar to those of corresponding primitive tumors, and the evaluation of platinum sensitivity in these preclinical models replicated the clinical environment of corresponding GSC patients (Arias-Diaz et al., 2023). The study showed that cell response to DNA damage stimulation is the main biological process associated with obtaining resistance to first-line treatment for GSC [100]. It can be seen that the organoid culture model in gynecology provides a valuable platform for studying the molecular processes that lead to uncontrolled cell proliferation and metastasis. This model can simulate the tumor microenvironment in the human body in more detail, including the interaction between tumor cells and surrounding tissues, thereby providing more accurate research results. Gynecological organoid culture models have higher biological reliability than traditional cell culture models (Francés-Herrero et al., 2022). This means that researchers can better understand how tumor cells grow and spread and study the molecular mechanisms that lead to this uncontrolled process. By gaining a deeper understanding of these mechanisms, scientists can identify new drug targets and develop more effective treatment strategies.

5.2.3.2 Research directions

As an emerging *in vitro* 3D modeling technology, organoid models reproduce the physiological and pathophysiological characteristics of the original tissues *in vivo* and are widely used in a variety of fields such as drug discovery, disease modeling, cancer research, developmental biology, regenerative mechanisms, precision medicine and organ transplantation. Tumors are not only aggregates of malignant cells but also well-organized complex ecosystems. The immune component within the tumor, called the tumor immune microenvironment, has long been shown to be closely associated with tumor development, recurrence, and metastasis (Fu et al., 2021). Therefore, more scholars have also initiated studies on the role of organoids in tumor immunotherapy.

Organoids can be co-cultured with immune cells. Zhou G. et al. established immune organoids by co-culture protocol of cholangiocarcinoma organoids and peripheral blood T cells and successfully mimicked effective anti-tumour immune response *in vitro* (Zhou et al., 2022). Chan I. S. et al. co-cultured exogenous immune cells directly with tumor epithelial cells premixed in matrix gel to simulate the interaction between immune cells and tumor cells without the need to establish tumor-like organs in advance (Chan and Ewald, 2022). In addition, organoid and tumor immunity can still be studied using air-liquid interface (ALI), and 3D microfluidics (Zheng et al., 2019; Esser et al., 2020).

Organoids have great potential for tumor immunotherapy development. TCR sequencing of peripheral blood T cells from pancreatic cancer organoid co-cultures revealed significant expansion of a specific subpopulation of T cell clones, while a model constructed from a mixture of organoids and cognate immune cells could also be used to induce peripheral blood T cell killing of tumor cells, demonstrating that the model is a viable platform allowing individual patient immune systems and tumor cells to be investigated in the context of an individualized immunotherapeutic responses while maintaining viability (Lin et al., 2023b). Jacob F. et al. report the generation and biopreservation of patient-derived glioblastoma-like organoids (GBOs) by co-culturing glioblastoma-like organoids with CAR-T cells, demonstrating the ability of CAR-T cells to specifically kill the target cells instead of completely eliminating all tumor cells, and that this co-culture model provides a viable approach to testing the efficacy of CAR-T therapies (Jacob et al., 2020).

The emergence and continuous development of organoids have become a new technology in anti-tumour immunotherapy research, with great potential for mimicking the effects of immunotherapy, investigating drug resistance mechanisms, and developing new combination therapies, which means that cutting-edge researchers can devote their attention to organoid immunotherapy as a point of intervention to promote the clinical translation of tumor immunotherapy, and ultimately help to achieve personalized immunotherapy.

5.2.3.3 Models of training

Owing to the rapid progression of most gynecological tumors and the fact that patients are often diagnosed in the middle and late stages, it is particularly important to develop an appropriate treatment plan. We can predict the response of patients to biopsy tissue treatment through organoids to select the most suitable treatment plan for patients (Lõhmussaar et al., 2020a). However, traditional organoid culture systems rely on manual operations, making the cultivation process more cumbersome. Additionally, individual and batch differences in culture also limit the transformation and application of organoids (Corrò et al., 2020). Therefore, we also need to find rapid methods for cultivating organoids to enable patients to receive personalized treatment as soon as possible. The emergence of organoids provides new development opportunities for research in fields such as disease modeling, drug screening, and tissue development (Rossi et al., 2018). The human organ chip simulates the structure, function, and microenvironment of human tissues or organs by constructing miniature tissues or organs on microfluidic chips. Therefore, using microfluidic chip technology to construct a high-throughput automated organoid culture system can greatly improve cultivation efficiency, reduce human errors and batch differences, and accelerate organoid transformation and application (Shirure et al., 2021). At present, according to the different target organs constructed, organ chips can be divided into lung chips (Kim et al., 2024), brain chips (Wang et al., 2018; Song et al., 2022), kidney chips (Lee et al., 2021), and intestinal chips (Hakuno et al., 2022) for drug screening and safety evaluation.

Therefore, with the advent of the digital era, the combination of gynecological tumor organoids and organ chips has become an important trend. The in-depth application of this technology in the field of gynecological tumors provides a high-quality platform for disease mechanism research, clinical diagnosis, and target drug discovery.

5.3 Limitations of the current study

Currently, there are still many issues facing research on gynecological malignancies and organoid studies. 1) Research is currently focused on different studies of organoid models, and although the research aspects show diversity, the experimental results lack reproducibility due to biological experimental materials, laboratories, researchers, and other factors. 2) The type of methodological research is more focused on the qualitative results of drug efficacy and disease models, and quantitative indicators based on organ-like gynecological malignant tumor targeting experiments at the level of various types of factors and protein expression levels need more practice and validation. 3) The Frontier Research Cellular Processes Direction lacks an in-depth exploration of molecular pathways that have been studied using organoids, especially common gynecological cancer-related signaling pathways. 4) In this study, only three types of gynecological tumor-like organs were reviewed in detail, i.e., OC-like organs, EC-like organs, and CC-like organs, and few types of tumors were studied.

Future research directions include the following. 1) Investigate the differences in organoid structure or genetic drift in transmission to enhance experimental reproducibility. 2) Highlighting the importance of quantitative results, conducting analysis of actual cell viability, proliferation rate, and other efficacy tests, transforming qualitative research dimensions into quantitative reality dimensions, improving the reliability of organoid application of oncology treatments, and appropriately the incorporation of positive and negative controls, the presence of adequate sample sizes, and the correct statistical test method to increase the accuracy of the assessment. 3) Based on the organ-like model, we will explore the role of common gynecological cancer-related signaling pathways such as PI3K/AKT/mTOR, p53, Wnt, and NF-κB in the progression of gynecological malignant tumors and drug resistance, so as to strengthen the depth of the research direction. 4) Based on OC, EC, and CC, we will carry out specific model construction of different types of other gynecological tumors from the perspective of organ-like, in order to improve the success rate of modelling, study the pathogenesis and therapeutic targets, and achieve the purpose of precision medicine.

6 Conclusion

The research described in this review mainly found that OC-like organs were mainly constructed by the matrix gel method, and some studies used the hydrogel method to construct organ-like structures. Both EC and CC organoids were modeled using the matrix gel method. By reviewing three types of gynecological tumors, we found that the organoids of OC, EC, and CC adequately summarize the genetic and morphological characteristics of primary tumors in various subtypes, which can be used for disease modeling, drug detection and screening, patient stratification, and drug response prediction. Modeling gynecological tumor diseases through organoids, replicating the mutation landscape of the primary tumor, and preserving the genomic map of the tumor from which it originated is useful in identifying genes related to OC progression, aiding in disease etiology research, and exploring the mechanisms of gynecological tumor occurrence and development. In addition, constructing different gynecological tumor-like organs can preserving the histological and genetic characteristics of the original tumor as well as tumor heterogeneity, evaluating the efficacy of individual drugs, or conducting drug screening experiments and drug sensitivity tests to explore the mechanisms of drug resistance and drug action in gynecological tumors. Molecular heterogeneity exists in gynecological tumors, leading to differences in drug reactions among different patients, requiring precision medicine. Therefore, organoids that can preserve the heterogeneity and genetic characteristics of the original tumor have advantages in precision medicine. Organ-like models taken from different patient tissues have different drug reactions, and there are broad prospects for stratifying individual patients into customized treatment plans. In summary, the main contribution of this study is the discovery that patient-derived organoids have clinical translational effects in predicting drug responses and resistance mechanisms, and have great potential in precision medicine in the future. Additionally, gynecological tumor organoids provide a choice for *in vitro* personalized simulation of source tissues and organs, which is conducive to promoting the development of clinical personalized precision medicine and regenerative medicine.

On this basis, we point out the dilemmas and difficulties in the existing research and application and propose future directions worthy of research from the perspectives of evaluating the reliability and accuracy of the application of organoids, enriching the types of gynecological tumors studied by organoids, and promoting the translation and application of the research results. It is worth pointing out that although we have recognized the importance of organoids in experimental studies of gynecological malignancies, knowledge of them is still limited, which constrains the development of organelle-based therapeutic agents and treatment modalities, and we need to further explore the optimal modeling methods of gynecological tumor-associated organoids and improve the success rate of modeling, so that it can be used to cultivate more kinds of gynecological tumor-associated organoid models for guiding clinical treatment.

Author contributions

YS: Formal Analysis, Funding acquisition, Project administration, Supervision, Writing–original draft,

Writing–review and editing. YW: Formal Analysis, Writing–original draft, Writing–review and editing. SW: Formal Analysis, Writing–original draft, Writing–review and editing. CL: Formal Analysis, Writing–original draft, Writing–review and editing. FH: Formal Analysis, Funding acquisition, Writing–original draft, Writing–review and editing.

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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.

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Glossary

CC	cervical carcinoma
EC	Endometrial cancer
OC	Ovarian cancer
3D	Three- dimensional
PARP	poly ADP-ribose
HRD	Homologous Recombination Deficiency
HGSOC	High-grade serous ovarian cancer
LGSOC	Low-grade serous ovarian cancer
OSE	ovary surface epithelial
COL1	collagen 1
NRG1	Neuregulin 1
SFK	SRC family kinase
ECM	extracellular matrix
FAK	Focal Adhesion Kinase
CDK	cyclin-dependent kinase
HRR	Homologous recombination repair
MCS	Multicellular spheroid
EHS	Engelbreth-Holm-Swarm
ECM	Extracellular matrix
ERRα	estrogen-related receptor α
PDX	patient-derived xenografts
Kras	Kirsten rat sarcoma viral oncogene homolog
CAF	Cancer associated fibroblasts
ASO	Antisense oligonucleotides
FGFR	Fibroblast Growth Factor Receptor
HPV	Human papillomavirus
SCNEC	Cervical small cell neuroendocrine carcinoma
SCCC	Cervical small cell carcinoma
cCCC	Cervical clear cell carcinoma
SqCa	squamous cell carcinoma
AdCa	Adenocarcinoma
SCJ	squamocolumnar junction
CESC	Cervical squamous cell carcinoma
LUM	Lumican
ACT	Adoptive T cell therapy
TIL	Tumor-infiltrating lymphocytes
PDTO	Patient derived tumor organoids
BME	Basement membrane extract
EV	Extracellular vesicles
GSC	Gynecological serous carcinoma

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Gastrointestinal tract organoids as novel tools in drug discovery

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Organoids, characterized by their high physiological attributes, effectively preserve the genetic characteristics, physiological structure, and function of the simulated organs. Since the inception of small intestine organoids, other organoids for organs including the liver, lungs, stomach, and pancreas have subsequently been developed. However, a comprehensive summary and discussion of research findings on gastrointestinal tract (GIT) organoids as disease models and drug screening platforms is currently lacking. Herein, in this review, we address diseases related to GIT organoid simulation and highlight the notable advancements that have been made in drug screening and pharmacokinetics, as well as in disease research and treatment using GIT organoids. Organoids of GIT diseases, including inflammatory bowel disease, irritable bowel syndrome, necrotizing enterocolitis, and *Helicobacter pylori* infection, have been successfully constructed. These models have facilitated the study of the mechanisms and effects of various drugs, such as metformin, Schisandrin C, and prednisolone, in these diseases. Furthermore, GIT organoids have been used to investigate viruses that elicit GIT reactions, including Norovirus, SARS-CoV-2, and rotavirus. Previous studies by using GIT organoids have shown that dasabuvir, gemcitabine, and imatinib possess the capability to inhibit viral replication. Notably, GIT organoids can mimic GIT responses to therapeutic drugs at the onset of disease. The GIT toxicities of compounds like gefitinib, doxorubicin, and sunset yellow have also been evaluated. Additionally, these organoids are instrumental for the study of immune regulation, post-radiation intestinal epithelial repair, treatment for cystic fibrosis and diabetes, the development of novel drug delivery systems, and research into the GIT microbiome. The recent use of conditioned media as a culture method for replacing recombinant hepatocyte growth factor has significantly reduced the cost associated with human GIT organoid culture. This advancement paves the way for large-scale culture and compound

Abbreviations: ASCs, adult stem cells; *CagA*, cytotoxin-associated gene A; CD, crohn's disease; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulato; CRC, colorectal cancer; EMT, epithelial-mesenchymal transition; EYTA, eicosatetraynoic acid; FAP-Cos, familial adenomatous polyposis - colonic rganoids; GIT, gastrointestinal tract; HIE, human intestinal enteroids; HIOs, human intestinal organoids; *H. plori, Helicobacter pylori*; PSC-Cos, colonic organoids derived from pluripotent stem cells; HuNov, Human Norovirus; IBD, inflammatory bowel disease; IFN-α, interferon-alpha; MIO, mouse intestinal organoid; PDOs, patient-derived organoids; PSCs, pluripotent stem cells; PSC-HIO, human intestinal organoids derived from pluripotent stem cells; SNPs, single-nucleotide polymorphisms; UC, ulcerative colitis.

screening of GIT organoids. Despite the ongoing challenges in GIT organoid development (e.g., their inability to exist in pairs, limited cell types, and singular drug exposure mode), these organoids hold considerable potential for drug screening. The use of GIT organoids in this context holds great promises to enhance the precision of medical treatments for patients living with GIT diseases.

KEYWORDS

organoids, gastrointestinal, drug discovery, disease modeling, precision medicine

1 Introduction

Organoids are organ-specific cell cultures developed from *in vitro* pluripotent stem cells (PSCs) or pluripotent adult stem cells (ASCs) to mimic the structure and function of their corresponding *in vivo* organ (Sato et al., 2009). These cells are cultured in specific *in vitro* environments to form tiny cell populations that self-organize and differentiate into functional cell types (Schutgens and Clevers, 2020). These organoids exhibit highly physiological properties that recapitulate the differentiation capacity of cells, tissue structures, as well as the interactions between cells and between cells and matrices (Günther et al., 2022). Gastrointestinal tract (GIT) organoids were first developed in Hans Clever's laboratory (Yi et al., 2021), where researchers successfully constructed intestinal organoids with intestinal crypt-villus structures *in vitro* by extracting Lgr5+ ASCs directly from the intestines and culturing them with appropriate growth factors and supportive substrates (Sato et al., 2009). Since the development of intestinal organoids, various types of organoid models, such as those for the esophagus, lung, liver, stomach, pancreas, and colorectum, have also been developed (Clevers, 2016). These models provide more accurate biological representations to aid research in areas such as immunotherapy, new drug discovery, and drug screening (Tang et al., 2022).

GIT organoids have advantages over two-dimensional (2D) cell systems and intestinal explant models. The primary drawbacks of 2D cell systems is that they lack many of the characteristics of normal GIT epithelium, they contain a single cell type, they lack the complex structure of *in vivo* tissues (Sato et al., 2009; Rossi et al., 2018), and they have a different genetic profile than normal cells (Millen et al., 2023; Sommerkamp et al., 2021). In contrast, intestinal exosomes reflect the complex structure of the *in vivo* intestinal tract, but they do not support passaging cultures (Sato et al., 2009; Xi et al., 2021). The development of GIT organoids remedies these deficiencies, and the model has numerous proven advantages (Sato et al., 2009). GIT organoids exhibit high similarity to the original tissue and can represent the patient cohort, capturing diversity. The high similarity between GIT organoids and the original tissue is reflected in aspects such as anatomical morphology, cellular composition, physiological function, and gene expression patterns. GIT organoids contain most intestinal epithelial cell types, including absorptive cells, cup cells, pan cells, and tufted cells, and have crypt-like structures and villous regions that are key in *in vivo* human intestinal tissue (Filippello et al., 2022; Kim et al., 2022). GIT organoid models can also be cultured for more than 1.5 years during which time they remain functional (e.g., the motility, absorption, and secretion functions of the gastrointestinal tract.) (Elbadawi et al., 2021). The gene expression pattern of the GIT organoid is more similar to that observed in normal tissues than in 2D cell system or animal models (Oberdoerffer et al., 2008; Legnini et al., 2023). This model provides advantages for studying genomic and epigenomic host-environment interactions (Witonsky et al., 2023). GIT organoids represent the patient cohort, capture diversity, and are manifested in the following aspects: First, GIT organoids represent the personalized characteristics of the patient's disease. GIT organoids derived from patient-specific induced pluripotent stem cells or directly from patient biopsy tissue enable them to represent the individual's genetic background, potentially capture the patient-specific disease characteristics, and are excellent models for studying intestinal epithelial interactions (Deleu et al., 2023). Second, GIT organoids capture the diversity of diseases. By creating organoids from patients with different diseases, researchers can study a range of pathologies and pathological processes, including inflammatory bowel disease, gastrointestinal cancer, *H. pylori* (*Helicobacter pylori*) infection, and viral infections. GIT organoids also allow patient-derived viruses to exist and replicate efficiently, circumventing the limitation that the use of laboratory-adapted strains is not representative of all circulating strains (Yin et al., 2015). Ultimately, GIT organoids also capture the diversity of populations. Biobanks of organoids from different populations have been established (Yao et al., 2020), ensuring that research is representative of a broad population and understanding how different genetic backgrounds affect disease susceptibility and treatment responses. Due to the high accuracy of GIT organoid disease modeling (Belair et al., 2020), it has also been used to predict patient responses to therapy (Aalbers et al., 2022) and subsequent clinical outcomes, thereby providing therapeutic guidance (Moussa et al., 2020).

GIT organoids have been increasingly used in disease research and drug screening, but there is a lack of literature that summarizes and discusses data around the use of GIT organoids as disease models and drug screening platforms. Herein, we focused on GIT organoid modeling of related diseases, and found that a variety studies have successfully been carried out using organoid-based GIT disease models. Examples include, pharmacological studies into clinical treatments, with some helping to elucidate the pathogenic mechanisms of GIT diseases and screening for preventive and therapeutic drugs. Further, previous studies using such organoid models have helped to reveal the pathology of viral infections and the subsequent GIT responses, leading to the development of novel drugs that inhibit viral replication. These models have also been key for testing the GIT toxicity of drugs and guiding their use in clinical practice. Owing to their use in wide range of applications and the richness of the data they can yield, GIT organoid models are important tools in disease research, drug screening, pharmacokinetics, drug toxicology research, and clinical practice,

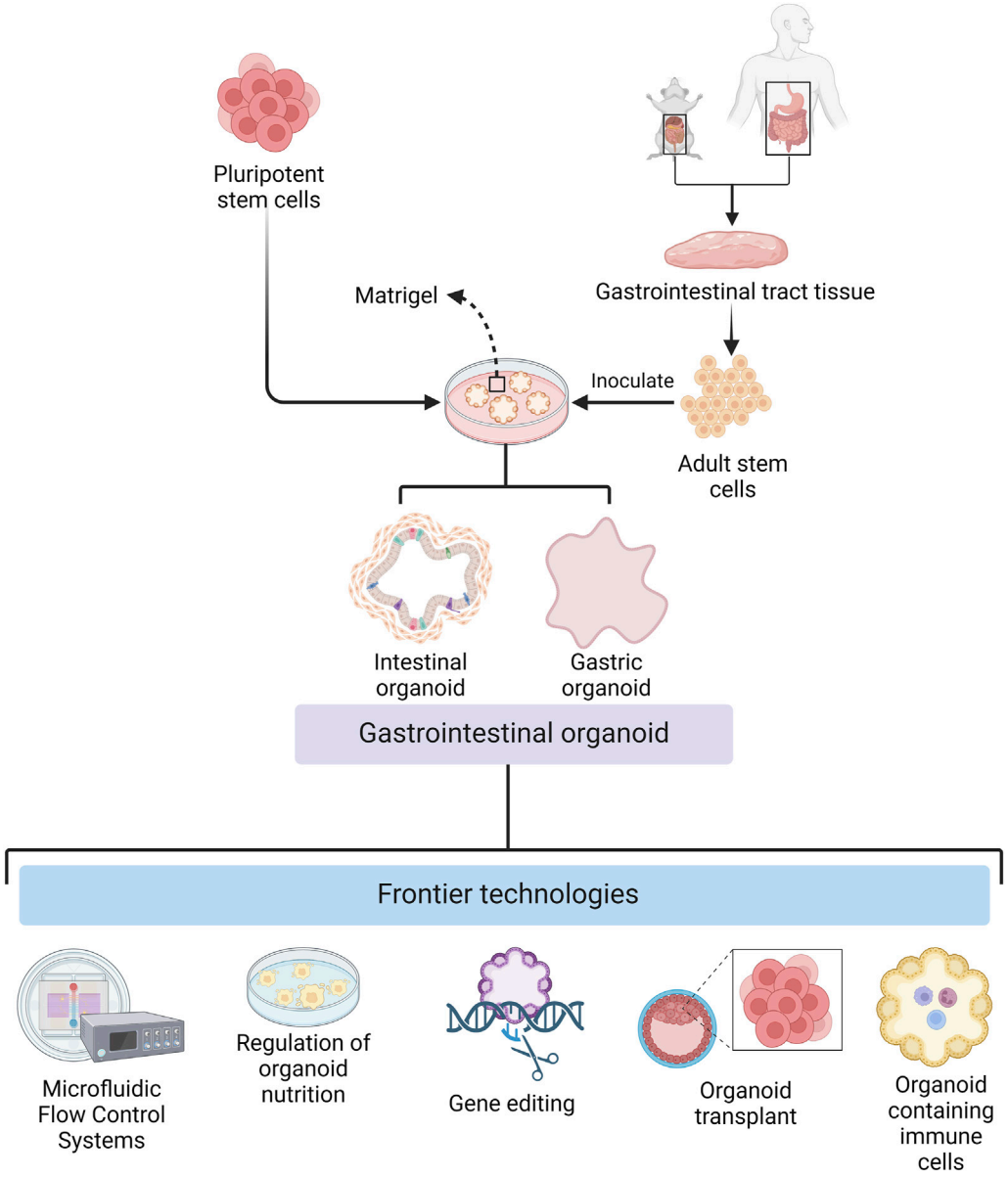

FIGURE 1
Cultivation of gastrointestinal organoids and frontier technologies (Created with BioRender.com).

among others, and promotes the development of precision medicine to improve global health.

2 Advances in organoid technology

There have been considerable developments in different aspects of organoid technology for basic research and clinical applications (Figure 1). For example, there have been breakthroughs in organoid culture technology, both in terms of broad applicability and specificity. In biomedicine, they have been transformed from ordinary *in vitro* models that can only be used for disease research to all-purpose models that can be used to study the mechanisms of viral and parasitic infections. The development of organoids that contain components of the immune system has further improved their utility as models for disease relevant and physiological *in vitro* research.

The development of organoid culture technology has promoted the wide application of organoids in basic research and clinical therapy. During organoid culture, cells can be attached to scaffolds composed of natural extracellular matrix or synthetic materials (Murakami and Masui, 1980; Giandomenico et al., 2019; Jaganathan et al., 2014; Garreta et al., 2021), or they can be aggregated to form microtissue spheroids by droplets, magnetic fields, or special synthetic materials. The use of collagen gels instead of matrix gels also makes low-cost mass replicable organoid models a reality (Takahashi et al., 2023), facilitates large-scale culture and complex screening of GIT organoids, and expands the application of GIT organoids in various research fields. The reduction in culture costs has been accompanied by an increase in the precision of

culture conditions. Wuputra et al. (2021) developed precise culture conditions for gastric organoids, which have improved the accuracy of GIT organoids as models for clinical therapeutic and medical applications.

In addition to the development of culture techniques suitable for general GIT organoids, modifying the culture conditions to incorporate disease-specific features enhances their suitability for disease research applications. Certain disease studies necessitate specific environmental conditions, such as controlled oxygen concentrations, which require techniques to regulate the oxygen environment of the organoids. Li et al. (2014) innovatively used an air–liquid interface culture method for modeling colorectal cancer (CRC) organoids, which improves *in vitro* oxygenation and enables studies of hypoxic diseases with control of this variable. Also, Zheng et al. (2021) designed a microfluidic chip capable of precisely regulating oxygen concentrations in each chamber. These studies provide technical support for organoid as a platform for researching hypoxic diseases. Moreover, certain diseases necessitate specific nutritional environments. Perlman et al. (2023) developed a new technique for culturing malnourished gastric organoids, which can alter the nutritional status of the organoids and provide a basis for the use of organoids as a research tool for studying the effects of nutritional status on the GIT epithelium.

While the application of organoids in disease research is expanding, its use as a therapeutic tool for clinical diseases is also becoming more widespread. Huang et al. (2023) differentiated cultured human gastric stem cells into islet-like organoids for the treatment of diabetes mellitus. Moussa et al. (2020) utilized organoid transplants to repair intestinal post-radiation injuries, which provided the basis for the development of organoid regenerative medicine. In addition, GIT system-on-a-chip organoids have emerged as promising *in vitro* models for preclinical studies. These advances are based on recent developments in several technologies such as bioprinting, microfluidics and organoid research.

Organoid technology is continually evolving for general disease research, but researchers are also developing new experimental models, combining organoids with gene editing techniques, and facilitating the study of virus and parasitic infection mechanisms. Kim et al. (2022) developed bovine gastric organoids as a novel *in vitro* model to study host-parasite interactions in GIT nematode infections. Gebert et al. (2024) utilized a gene-encoded calcium indicator for real-time calcium imaging of virus-infected organoids to establish an adaptable platform to represent cellular signals in virus-infected GIT nematodes. Moreover, an encoded calcium indicator has also been used for real-time calcium imaging of virus-infected organoids, establishing a tractable method for characterizing cellular signals in virus-infected GIT organoids.

Organoid modeling has great potential in biomedicine; however the lack of a model immune system in these models is a major drawback (Günther et al., 2022). However, by transplanting PSC-derived human intestinal organoids (HIOs) under the kidney capsule of mice with a humanized immune system, Bouffi et al. found that human immune cells temporarily migrate to the mucosa and form cell aggregates similar to human intestinal lymphoid follicles. In addition, upon exposure to microorganisms, the number of epithelial microfollicular cells in this study increased, leading to immune cell activation and secretion of immunoglobulin A antibodies in the lumen of the HIOs. This immune cell-containing HIO system provides a framework for future studies of infection- or allergen-driven intestinal diseases (Bouffi et al., 2023), compensating for the lack of an immune system in organoids. Taken together, the continued advances in organoid technology are aided by the available comprehensive supporting methods, making it a valuable tool for biomedical research.

3 Models for GIT disease

3.1 GIT organoids as tools in inflammatory bowel disease (IBD) research

GIT organoids are excellent models which have been used to study a variety of GIT diseases (Figure 2), including IBD (Holmberg et al., 2017) [i.e., Crohn's disease (CD) and ulcerative colitis (UC)]. IBD manifests itself as recurrent episodes of inflammation and remission, all characterized by chronic inflammation in different parts of the GIT tract. These inflammatory episodes can cause diarrhea, abdominal pain, blood in the stool, and other symptoms. Although IBD pathogenesis remains unclear, previous studies have shown that immune dysfunction in the intestinal mucosa due to genetic and immunological factors plays an important role in IBD pathology (Iacucci et al., 2024).

The influence of genetic factors on IBD pathogenesis has been demonstrated by twin, targeted sequencing, and genome-wide association studies (GWAS) (Mokry et al., 2014). In 2013, an organoid-based genetic study of IBD was conducted by researchers who generated acetylated histone 3 lysine 27 profiles from primary intestinal epithelial cells and subsequently cultured organoid derived from these cells. From this, 92 out of 163 IBD-associated single-nucleotide polymorphisms (SNPs) were shown to be associated with differentially active regulatory elements. Moreover, variations in these SNPs were shown to create or disrupt known binding motifs, suggesting that they may affect the binding of transcriptional regulators, thereby altering the expression of regulated genes. In addition to variants in protein-coding genes, variants in noncoding DNA regulatory regions active in intestinal epithelial cells and immune cells may also be involved in IBD pathology (Mokry et al., 2014). With the wide study of GWAS' and SNPs, many genes involved in immune regulation have been successfully identified as susceptibility genes for IBD, including Interleukin 28, which controls intestinal epithelial cell proliferation in mice with colitis and accelerates mucosal healing by activating the signal transducer and activator of transcription 1 protein (Chiriac et al., 2017).

Patients with IBD suffer from an imbalance in immune regulation, and the marked differences in the immune microenvironment between the two models of IBD (i.e., UC *versus* CD), are also reflected in T cell differentiation patterns (Biton et al., 2018). In organoid models of UC and CD, T cells either differentiate more into the Th17 or Th1 type, respectively. Interestingly, Hammoudi et al. demonstrated for the first time that autologous mucosal T cells can directly induce epithelial cell death in patients with CD (Hammoudi et al., 2022).

In conclusion, GIT organoids are important *in vitro* models for IBD research, where they are used to accurately simulate the effects

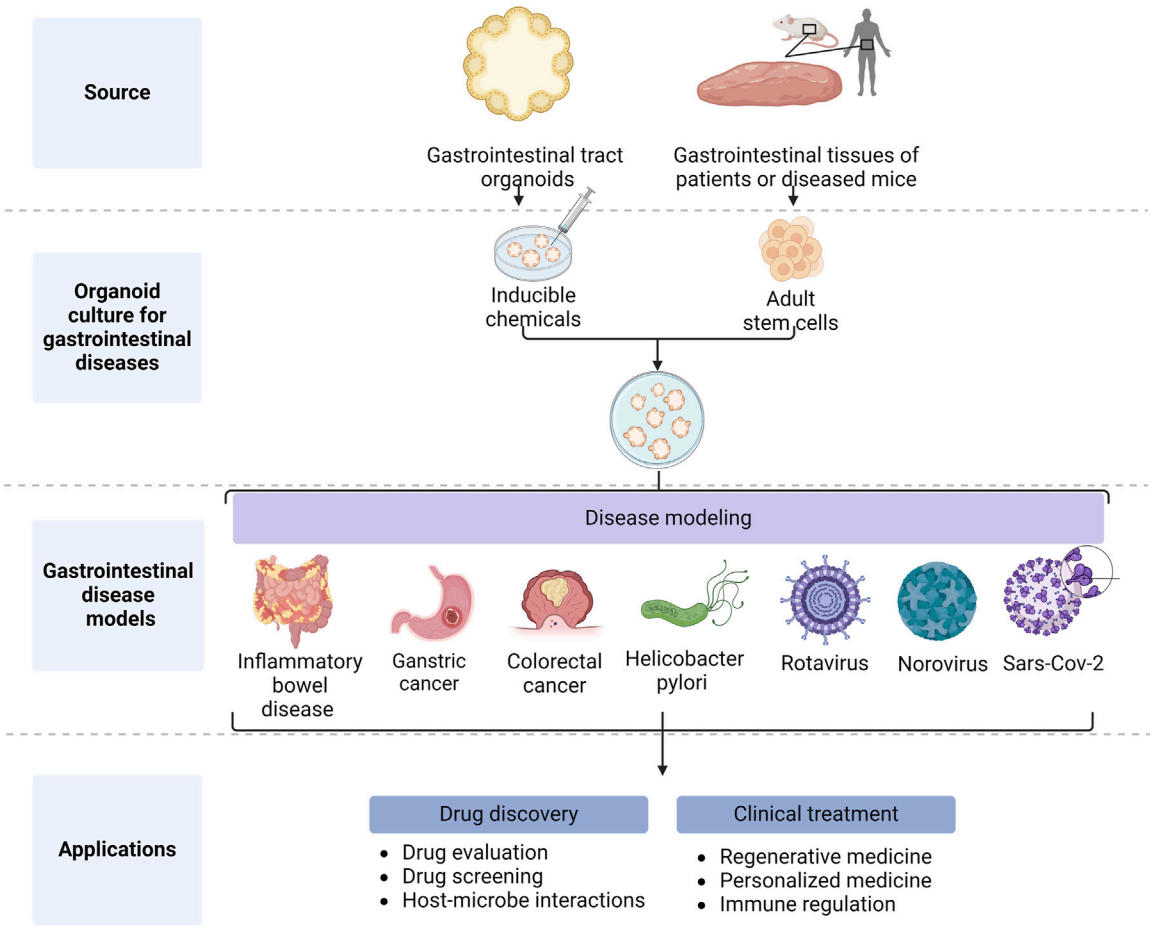

FIGURE 2
Application of gastrointestinal organoids (Created with BioRender.com).

of reductive genetic and immune factors on intestinal epithelial damage in IBD.

3.2 GIT organoids as tools in GIT cancer research

GIT tumor organoids provide an ideal *in vitro* model for studying GIT tumor cells and molecular signaling pathways. These organoids are able to maintain the complexity of GIT tumor cells and can recapitulate tumor biology.

Many studies have been carried out using organoids to mimic gastric cancer progression. Further, the combination of GIT organoids and gene editing technology has facilitated basic research into gastric cancer. Tong et al. induced normal gastric organoids to develop the malignant phenotype of gastric cancer by knocking down the tumor suppressor, *CDH1*, and they subsequently showed that knocking down *RHOA* restored them to their normal morphology (Tong et al., 2023). These data suggested that normal gastric organoids can develop into gastric cancer organoids, and their malignant behavior can be reverted to normal with relevant interventions, highlighting the importance of organoids in studying tumorigenesis and development. In patients with GIT tumors, gastric cancer peritoneal metastasis (GCPM) is a leading cause of death. During this process, monocyte-like dendritic cells (DCs) with pro-angiogenic effects are increased and their antigen-presenting capacity is reduced. In addition, gastric cancer clusters with high plasticity have shown a tendency to transition to a high-proliferative phenotype through an autophagy-dependent plasticity program, whereas autophagy inhibitors induced apoptosis in patient-derived organoid (PDO) (Huang et al., 2023). These findings provide insights into the developmental trajectories of cancer and immune cells that underlie GCPM. GIT organoids can be used to recapitulate the entire process of gastric cancer occurrence, development, and metastasis, making them excellent models for gastric cancer research.

In intestinal tumors, Notch signaling plays an important role in regulating tumor progression and metastasis, and it is associated with poor prognosis of CRC intestinal epithelial–mesenchymal transition (EMT). Using intestinal organoids to understand how the Notch pathway regulates epithelial cell regeneration and differentiation is essential for studying intestinal stem cell homeostasis and pathogenesis in intestinal tumors. Fujii et al. (2016) found that Notch signaling activation in organoids alone was not sufficient to induce CRC development. Heuberger et al. (2021) demonstrated that when Notch signaling activation was

accompanied by inhibition of p53, it promotes EMT and subsequent tumorigenesis (Chanrion et al., 2014). These studies suggest that GIT tract-like organoids accurately represent physiological signaling during intestinal tumorigenesis, making them valuable tools in studies of intestinal tumor pathogenesis.

3.3 GIT organoids for *Helicobacter pylori* studies

H. pylori is a major cause of gastric diseases and tumors. The attachment, colonization, and cytotoxin-associated gene A (*CagA*) transport that characterize *H. pylori* infection have been reproduced in organoids. Recognition and attachment of *H. pylori* to target cells is important for its infection and cellular reprogramming. Confocal live cell microscopy has been used to visualize *H. pylori* attachment to infected gastric organoids from normal human mucosa. Based on this, Aguilar et al. used organoids to study *H. pylori* attachment characteristics and found that *H. pylori* preferentially adhered to highly differentiated depressed cells marked by high levels of Gastrokine 1, Gastrokine 2, and the prostate stem cell antigen. Further, this study also showed that attachment was not associated with the expression of Mucin-5AC or the prostate stem cell antigen, but rather depended on TlpB-dependent chemotaxis of the bacterium in response to ureides released by the host cell (Aguilar et al., 2022).

Using organoid models, Sigal et al. (2015) found that *H. pylori* colonizes and manipulates progenitor and stem cell compartments, altering metabolic dynamics and glandular proliferation. This finding has important implications for GIT stem cell biology and *H. pylori*-induced gastric pathology. Furthermore, infection with *H. pylori* strains infused with *CagA* is a major risk factor for death from gastric cancer (Zhang et al., 2022). This strain causes gastric epithelial cell transformation by promoting EMT, which disrupts junctions and enhances the motility and invasiveness of infected cells. This was confirmed in monolayers of cells derived from human organoids, suggesting that *H. pylori* is generally able to transfer *CagA* into organoid cells.

3.4 GIT organoids can mimic viral infections

GIT organoids allow patient-derived viruses to exist and replicate efficiently with high accuracy in their disease modeling (Belair et al., 2020; Yin et al., 2015), thereby providing a powerful model system for studying virus-host interactions. These GIT organoids have been used in the study of viral infections capable of causing GIT reactions, including diarrhea [e.g., rotaviruses, Human Norovirus (HuNoV), and SARS-CoV-2] due to their location and functional specificity.

Rotaviruses primarily infect the ileum and jejunum of the host and are capable of destroying enterocytes and impairing intestinal absorption. While we know that rotavirus non-structural protein 4 (NSP4) stimulates intestinal secretion and activates the enteric nervous system, thereby inducing diarrhea, little else is known about the mechanisms behind its pathology. The development of *in vitro* models of the GIT tract will likely be crucial for the study of rotavirus pathogenesis. Finkbeiner et al. used HIOs for the first time for *in vitro* culture of rotaviruses and observed efficient replication of the viruses by immunofluorescence microscopy (Finkbeiner et al., 2012). These data suggest that intestinal organoids are a suitable model for to study rotaviruses.

HuNoV is also an important pathogen in acute gastroenteritis. The development of the HIO culture system provides a new model for further study of HuNoV infection, signaling and pathogenesis. Ettayebi et al. (2016) simulated HuNoV infection in an intestinal organoid model and using immunofluorescence analysis and electron microscopy they confirmed the replication process of this virus in intestinal organoids. Importantly, their data showed that the virus proliferated in the model to produce intact virus particles. This study provides an important experimental model for further research into HuNoV pathology.

Nearly half of patients with SARS-CoV-2 experience GIT symptoms such as diarrhea or nausea, and it is thought that SARS-CoV-2 infection elevates proinflammatory factors that lead to intestinal inflammation (Guo et al., 2021). Krüger et al. (2021) detected virus spiking (S) proteins in 10% of the cells of intestinal organoids after 24 h exposure to SARS-CoV-2, which increased to 57% after 48 h, suggesting virus replication and transmission, which was confirmed by the detection of nucleocapsid (N) proteins. These results suggest that SARS-CoV-2 can effectively infect and replicate in intestinal organoids.

4 Drug screening for GIT diseases

Traditionally, most therapeutic drug screening and research campaigns for GIT diseases have been conducted using 2D cell systems. However, there are now an increasing number of studies using GIT organoids for drug screening (Table 1). Compared to 2D cellular systems, GIT organoids provide a more complete picture of the GIT tract at the time of disease, thus increasing the accuracy of drug screening and giving the best options for disease prevention and treatment.

4.1 IBD treatment and prevention

IBD pathology is associated with intestinal tight junction disorders. Treatment options include targeted therapies such as steroids, aminosalicylates, and tumor necrosis factor-alpha (TNF-α) neutralizing antibodies, but many patients are insensitive to these therapies. Therefore, there is an urgent need for more effective treatments and drugs. An increasing number of studies have shown that intestinal epithelial damage organoids can effectively be used to screen for therapeutic drugs for IBD. In terms of using organoids in identifying preventive measures, Guo et al. (2023) found that excessive intake of (epi) catechins potentially damages the intestinal epithelium in mouse inflammatory intestinal organoids, which may increase the risk of intestinal damage. Thus, limiting or avoiding such risk factors could be an effective preventive measure for IBD.

Intestinal organoid modeling has also been used to highlight reduced intestinal epithelial permeability as a pathological marker of IBD, and therefore, a feature that can be addressed in IBD treatment. Inflammatory cytokines contribute to reduced epithelial permeability, and Hahn et al. used an organoid model of intestinal epithelial injury and found that metformin decreased

TABLE 1 Drug screening for disease (Sort by order of appearance in the article).

Disease	Agents	Dosage	Effect to gastrointestinal organoids	References
IBD	Schisandrin C	5–40 μM	Improves intestinal permeability Enhanced epithelial barrier formation	Kim et al. (2022)
UC	High-concentration acetate	100 mM	Protects intestinal barrier Anti-inflammatory	Deleu et al. (2023)
IBD	(Epi) catechin	0.03–3 mM	Damage to the intestinal epithelium	Guo et al. (2023)
IBD	Metformin	1 mM	Anti-inflammatory and improves intestinal permeability	Hahn et al. (2024)
IBD	*Lactobacillus reuteri*	100 μM	Repairing intestinal damage Maintaining intestinal epithelial regeneration and homeostasis	Wu et al. (2020)
UC	KAG-308	3 mg/kg qd	Anti-inflammatory Promotes regeneration of intestinal epithelial cells Enhances mucus production	Nishimura et al. (2019)
CD	EYTA Butyrate	50 μM 10 mM	Suppression of ECM genes associated with stenosis Suppression of collagen content and tissue stiffness	Jurickova et al. (2022)
CD	Spironolactone	0–250 μM	Blocking the organoid fibrosis response	Rodansky et al. (2015)
CD	Prednisolone	10 μM	Preventing barrier dysfunction	Xu et al. (2021)
HP	lapatinib Cherry-CAP	20 μM NA	Decreased the survival of *H. pylori* in infected organoids	Buti et al. (2020)
Rotavirus	IFN-α Ribavirin	1,000 IU/mL 10 μg/mL	Inhibition of rotavirus replication	Yin et al. (2015)
SARS-CoV-2	Remdesivir	0–100 μM	Inhibition of SARS-CoV-2 replication	Krüger et al. (2021)
Rotavirus	Gemcitabine	0–10 μM	Inhibition of rotavirus replication	Chen et al. (2020)
HuNoV	Ephedra herba	12.5–50 μg/mL	Inhibition of HuNoV replication	Hayashi et al. (2023)
HuNoV	Dasabuvir	5–20 μM	Inhibition of HuNoV replication	Hayashi et al. (2021)
HuNoV	Nitazoxanide	0.1–10 μg/mL	Inhibition of HuNoV replication	Dang et al. (2018)
SARS-CoV-2	Imatinib Mycophenolic acid Quinacrine Dihydrochloride	0.01–100 μM	Inhibition of SARS-CoV-2 replication	Han et al. (2021)
CRC	GANT61 DAPT ATO	10 μM	Enhances 5-Fluorouracil's chemosensitivity Inhibits invasiveness	Citarella et al. (2023)
CRC	Aspirin	0.5–2 mM	Rescue of the wnt-driven cystic organoid phenotype	Dunbar et al. (2021)
CRC	Butyrate	1 mM	Enhance the efficacy of radiotherapy Protecs the normal mucosa	Park et al. (2020)
GC	55 drugs	NA	Inhibition of tumor cell proliferation	Vlachogiannis et al. (2018)
GC	37 drugs	NA	Inhibition of tumor cell proliferation	Yan et al. (2018)
CRC	83 drugs	NA	Inhibition of tumor cell proliferation	Van De Wetering et al. (2015)
CRC	XAV939 Rapamycin	5 μM 10 μM	Reduce abnormal proliferation	Crespo et al. (2017)
CF	ELX-02	0–160 μM	Restore the CFTR function	Crawford et al. (2021)

inflammatory cytokine levels and restored intestinal epithelial permeability (Hahn et al., 2024). Intestinal epithelial permeability is also affected by associated proteins, and Kim et al. (2022) used an intestinal organoid assessment to show that Schisandrin C improved abnormal intestinal permeability and also regulated the expression of proteins closely associated with the development of leaky gut symptoms and IBD, as well as inflammation-associated proteins. Other inducible factors also contribute to intestinal inflammation, Zhang et al. showed that silencing melatonin receptor 1 A inhibited melatonin-induced inflammation in intestinal organoids (Xi et al., 2021). The above drugs play a role in the treatment of IBD by decreasing the levels of inflammatory cytokines, modulating inflammation-related proteins, and inhibiting intestinal epithelial damage-inducing factors, making them important candidates for

IBD treatment. In addition to reducing the effects of inflammation-related factors on intestinal inflammation, maintaining intestinal epithelial stability is also crucial for IBD treatment. Wu et al. used intestinal organoids simulating IBD barrier damage to show that *Lactobacillus reuteri* effectively maintains intestinal epithelial cell regeneration and homeostasis and repairs intestinal damage after pathological injury (Wu et al., 2020). These data provide important insights for the development of future UC and CD treatments, which are generally directed at epithelial damage and intestinal fibrosis. The recent advances in organoid technology allow for more accurate predictions of drug effects as well as aiding drug discovery and development. Deleu et al. (2023) used organoids derived from patients with UC to show that high-concentration acetate upregulated *HIF1α, MUC2,* and *MKI67*, while also downregulating most proinflammatory cytokines, which had a protective effect on epithelial resistance, barrier gene expression, and inflammatory protein production. Together, this suggests that high concentrations of acetate are effective therapeutic agents for UC. Nishimura et al. evaluated the therapeutic effects of the drug on intestinal epithelial cells using a colonoid organoid model and showed that the study drug KAG-308 inhibited immune responses and promoted the shift of cellular differentiation towards the secretory profile (Nishimura et al., 2019), suggesting that the KAG-308 could also be a candidate for UC therapy. HIOs provide a platform for testing personalized therapies for CD including small molecule drugs. Jurickova et al. (2022) tested the modulation of mitochondrial and wound healing functions associated with stricturing behavior by small molecules including eicosatetraynoic acid (ETYA) through CD-induced HIOs, and the results showed that in the HIO model ETYA modulated the stenosis-related ECM genes and suppressed collagen content and tissue stiffness, restored mitochondrial function, and promoted wound healing, suggesting a therapeutic effect of ETYA on CD. Rodansky et al. (2015) used HIO as a new model of intestinal fibrosis in CD. The results showed that spironolactone treatment blocked TGFβ-induced fibrosis in HIOs, suggesting that spironolactone can be used to treat CD. Xu et al. (2021) explored the effect of prednisolone on the intestinal-derived organoid epithelial barrier in CD patients and its mechanism, and found that prednisolone played a direct preventive role in cytokine-induced barrier dysfunction by regulating the expression of Claudin-2, E-cadherin, and immunoglobulin-like domain-containing receptor 1. The above drugs were preliminarily screened for their therapeutic effects on UC and CD through GIT organoids, providing additional therapeutic options for clinical treatment.

4.2 Drug development for GIT cancer

Chemotherapy and radiotherapy are the main means of CRC treatment, and the use of GIT organoids to simulate the GIT tract conditions in this period provides a powerful model for studying the mechanism of action of traditional drugs in oncology treatment, enhancing chemotherapy sensitivity, improving radiotherapy efficacy, and suppressing the adverse effects of radiotherapy, as well as screening for new therapeutic agents.

Aspirin has been shown to be a chemoprotective agent in the treatment of CRC, but its mechanism of action is not fully understood. Dunbar et al. (2021) used intestinal organoids and found that aspirin restored the Wnt-driven stem cell-like phenotype in HIOs. In addition, 5-Fluorouracil is the main chemotherapeutic agent for CRC but emergence of resistance limits its clinical use. Using GIT tumor organoids, Citarella et al. (2023) found that 5-Fluorouracil promotes mesenchymal cell proliferation and thus invasive phenotypes in *KRAS- and BRAF*-mutant organoids and can be used in combination with Hedgehog/GLI and Notch pathway inhibitors, as well as with GANT61 or arsenic trioxide (ATO) to restore chemosensitivity, suggesting that ATO and GANT61 are promising chemosensitizers in CRC. This study shows the promise of 5-Fluorouracil in lifting clinical therapeutic limitations. These two drugs have been further characterized in organoid-based studies, confirming their roles as effective agents for chemotherapy in CRC and demonstrating the value of GIT organoids as research models for drug improvement.

Radiotherapy is the other mainstay of treatment for GIT tumors, and the use of GIT organoids to improve the efficacy of radiotherapy and reduce radiotherapy-related injury is critical in the treatment of GIT tumors. Park et al. (2020) used organoids derived from patients with CRC to assess their response to radiotherapy, and found that butyrate does not increase radiation-induced cell death and improves regeneration of normal organoids and tissues after radiation. This study suggests that butyrate improves the efficacy of radiotherapy while protecting normal mucosa, a potential strategy to minimize radiotherapy-related toxicity.

In addition to small-scale drug screening for GIT tumors, GIT organoids have also performed well in large-scale drug screening for GIT tumors. Vlachogiannis et al. (2018) studied treatment responses of metastatic GIT cancers using PDOs and screened 55 drugs in these PDOs. They found that PDOs predicted responses to targeted or chemotherapeutic drugs with a sensitivity of 100% and a specificity of response was 93%. These data suggest that PDOs can be used in functional genomics to model tumors and conduct clinical trials. Further, Yan et al. (2018) screened 37 drugs in gastric tumor organoids and found that drugs such as pabukasin (Napabucasin), abemaciclib, an ataxia telangiectasia, and Rad3-related (ATR) kinase inhibitor (VE-822) could be candidates for gastric cancer treatment. van de Wetering et al. (2015) screened 83 drugs using CRC organoids and confirmed the link between drug resistance and genetic mutations. While drugs for GIT tumor therapies are being screened on a large scale for adenomatous polyposis, drug screening for adenomatous polyposis is also underway. Crespo et al. used colonic organoids (COs) as a platform for drug testing and showed that compounds XAV939 and rapamycin reduced proliferation of familial adenomatous polyposis colonic organoids (FAP-Cos). This study also identified a ribosome-binding antibiotic that effectively targeted aberrant WNT activity and specifically restored normal proliferation in *APC*-mutant familial adenomatous polyposis COs (Crespo et al., 2017). This study provides additional lead drug molecule candidates for the treatment of adenomatous polyposis.

4.3 *H. pylori* treatment

Helicobacter pylori is a major risk factor for gastric cancer, and through GIT organoid studies Buti et al. (2020) found that apoptosis-stimulating protein of p53 2 (ASPP2), a tumor

suppressor and important target of *CagA*, contributes to the survival of *CagA*-positive *H. pylori* in the lumen of infected gastric organoid tissues and that it is a key protein in disrupting cell polarity. Studies have shown that inhibiting CagA-positive *H. pylori* ASPP2 signaling with inhibitors of the epidermal growth factor receptor signaling pathway or specific peptides, prevents loss of cell polarity and reduces *H. pylori* survival in infected organoids. These findings suggest that maintaining the host cell polarity barrier reduces the deleterious consequences of *H. pylori* infection, thereby presenting a novel potential strategy for treating *H. pylori* infection.

4.4 Drug-mediated inhibition of viral replication

Viral infections often cause acute GIT reactions that can be severe and life-threatening. Rotavirus infection is usually acute and self-limiting, and it can cause chronic infections and serious illness in immunocompromised patients. Gemcitabine is a widely used anticancer drug. Chen et al. (2020) used HIOs to show that gemcitabine is an effective inhibitor of rotaviral infection, which is also beneficial for patients with cancer infected with this virus. In addition, Yin et al. (2015) found that interferon-alpha and ribavirin inhibit rotavirus replication through intestinal organoid studies. These studies suggest that intestinal organoids can be used to evaluate and screen for antiviral drugs.

HuNoV is a major cause of acute gastroenteritis and foodborne illness. GIT organoids can be used to screen for effective drugs to inhibit this virus. Hayashi et al. (2023) screened components of a Japanese–Chinese herbal medicine using stem cell-derived HIOs and found that ephedra herb considerably inhibits HuNoV infection among 22 herbs. Hayashi et al. (2021) used a human intestinal enteroids culture to screen a library of antiviral compounds using this system and successfully identified dasabuvir as a novel anti-HuNoV inhibitor. Using GIT organoids, other teams have found that thiazoles effectively inhibit HuNoV. Dang et al. (2018) discovered that thiazoles inhibit HuNoV replication by inducing the antiviral effector, Interferon regulatory factor-1.

SARS-CoV-2 still poses a serious risk to global health, with up to 50% of patients experiencing GIT symptoms such as diarrhea or nausea. Potent drugs to inhibit the replication of this virus are a key global public health concern. HIOs derived from pluripotent stem cells (PSC-HIOs) as well as colonic organoids (PSC-COs) are important tools for the identification of potent agents against SARS-CoV-2. Krüger et al. (2021) found that rameltegravir effectively inhibits SARS-CoV-2 infection and restores PSC-HIO morphology. Han et al. (2021) used hPSC-COs to perform a high-throughput screening of FDA-approved drugs and identified SARS-CoV-2 inhibitors including imatinib, mycophenolic acid, and quinacrine hydrochloride. Taken together, GIT organoids are clearly important drug screening tools for antiviral drugs, particularly for GIT indications.

4.5 Pharmacokinetic and drug toxicology studies

GIT organoids have been cultured to serve as *in vitro* models for exploring drug metabolism, and drug GIT toxicity studies (Lu et al., 2017; Park et al., 2019). Yamada et al. investigated the effects of a novel synthetic lithocholic acid derivative, Dcha-20, with vitamin D activity, on the expression of pharmacokinetic genes in HIOs, and found that Dcha-20 promotes the activity of the intrinsic defense system of intestinal epithelial cells (Yamada et al., 2022). In disease treatment, the decreased activity of the intestinal epithelial defense system makes it more difficult for the body to resist drug-induced GIT toxicity and symptoms such as vomiting and diarrhea. Therefore, research into *in vitro* models of GIT tract toxicity and prediction of drug GIT toxicity is necessary. Belair et al. (2020) used human GIT mesenchymal stromal tumor organoids to show that the model reproduced clinical drug-associated diarrhea with an accuracy of 90%, making it a suitable *in vitro* model for addressing the drug GIT toxicity during preclinical development. The molecular mechanisms of drug-induced GIT toxicity are increasingly being elucidated using GIT organoid models. This is the case for drugs such as gefitinib and Adriamycin (Rodrigues et al., 2022b; Rodrigues et al., 2022a). Further, Lu et al. (2017) used crypt organoid studies to demonstrate that the severe enterotoxicity of the anticancer precursor drug camptothecin-11 originated from the *UGT1A1*-dependent insufficient glucuronidation of its active metabolite, SN-38. Using a mouse intestinal organoid (MIO) model Wang et al. investigated toxicity molecular mechanisms behind the marine toxins, okadaic acid, and conotoxin (CgTx), and found that OA reduced cellular metabolism and energy production by affecting MIO cell transcription, ultimately leading to cell death. In contrast, CgTx upregulates intracellular hormone metabolism pathways by affecting the nuclear receptor pathway of MIO, leading to cell death and high energy production (Wang et al., 2022). An *in vitro* toxicological study of sunset yellow (SY) using an intestinal organoid model by Kong et al. (2021) found that SY disrupts homeostasis in intestinal epithelial cells by producing high levels of the endoplasmic reticulum stress and oxidative stress, and that long-term sustained consumption of SY may increase the risk of intestinal inflammation. Takahashi et al. (2023) developed a lower-cost intestinal organoid and found that YC-1 [3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole] induces apoptosis through the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway. It is the high degree of restoration of the physiological structure and function of the GIT tract that has led to the use of GIT organoids in a wide range of toxicological studies.

5 GIT organoids in microbiology research and clinical trials

GIT organoids are not only a platform for screening drugs for GIT diseases, but also play a role in immunomodulation, gut microbiota research, and the treatment of post-radiation intestinal epithelial damage, cystic fibrosis (CF), and diabetes. Importantly, GIT organoids also have an immunomodulatory role, Zhang et al. (2021) isolated extracellular vesicles using mouse and human GIT organoids and found that EVs play a crucial role in maintaining homeostasis in the host. The gut microbiota plays an important role in the formation of the intestinal immune system, and it has been found that in HIOs, 13-hydroxy-cis-6,cis-9-octadecadienoic acid (γHYD), and 13-oxo-cis-6,cis-9-octadecadienoic acid (γKetoD) produced by

Lactobacillus intestinalis, which are naturally occurring peroxisome proliferator-activated receptor delta ligands in the intestinal tract, are able to promote the β-oxidation of fatty acids and to reduce the accumulation of intracellular triglycerides, in order to improve lipid metabolism of the human intestinal tract (Noguchi et al., 2022).

GIT organoids are widely used in clinical trials and have been used as an important tool in regenerative medicine and as a medication guide for clinical treatment. Radiation therapy is commonly used for GIT tumors, and high-dose radiation exposure induces GIT stem cell death, leading to intestinal mucosal denudation and GIT syndrome death. In recent years, drugs to attenuate radiotherapy injury have been screened through intestinal organoid studies, and experiments using intestinal organoid transplantation cells to treat radiotherapy injury have been successful. Wang et al. (2020) used intestinal injury organoids and found that present arachidonic acid activated radiation-resistant Musashi-1+ cells promote intestinal epithelial repair. Fu et al. (2021) used intestinal organoids and found that knockdown or drug inhibition of sirtuin1 increased p53 acetylation and led to p53 stabilization, which considerably improved the survival of irradiated intestinal epithelial cells, suggesting that sirtuin1 inhibitors are an effective clinical countermeasure to attenuate intestinal damage caused by radiation exposure. Moussa et al. (2020) found that *in vitro* expanded epithelial cells transplanted from mouse colonoid organoids implanted, proliferated, and differentiated in irradiated mucosa and reduced ulcer size. This study demonstrates the potential of organoids to limit the effects of late radiation on the colon and opens the prospect of a combined strategy to improve their expansion capacity and therapeutic efficacy.

In a therapeutic study of CF, researchers used PDOs derived from patients with the *G542X* genotype and found that ELX-02 targeting of the *G542X* cystic fibrosis transmembrane conductance regulator (CFTR) nonsense allele restored CFTR function in HIOs (Crawford et al., 2021), supporting the clinical evaluation of ELX-02 as a through-putting agent for the treatment of CF caused by mutations in the *G542X* allele. Organoids are not only used to investigate CF therapeutic agents but also to guide the treatment for patients with clinical CF. Forskolin-induced swelling of patient organoids was used to measure patient-specific CFTR function and CFTR modulator response and has been used to clinically guide the treatment of a patient with a rare genotype of CFTR mutation (Aalbers et al., 2022).

GIT organoids can also be used in diabetes treatment research, supporting the discovery of diabetes therapeutic targets and the restoration of glucose homeostasis *in vivo*. Filippello et al. (2022) used MIOs that mimic lipotoxicity to find that lipotoxicity affects the differentiation of specific intestinal cell types in the intestinal tract, and also identified new targets related to the molecular mechanisms affected by lipotoxicity that may be important for the treatment of obesity and diabetes. Huang et al. (2023) cultured islet organoids differentiated from human gastric stem cells containing gastric insulin-secreting cells with similar molecular characteristics and function to β-cells. The organoids were found to acquire glucose-stimulated insulin secretion within 10 days and to restore glucose homeostasis in diabetic mice within 100 days post-transplantation, providing a potentially promising new approach to diabetes treatment.

6 Summary and outlook

In this review we discuss the application of GIT organoid technology in disease research and drug screening. GIT tract organoids maintain the genetic properties, physiological structure, and function of the GIT tract, have the ability to accurately model GIT diseases, and have great potential for aiding our understanding of disease pathology and for developing new treatments for GIT diseases. Due to their miniaturization and ability to mimic the physiological structure and function of the GIT tract, coupled with their amenability to high-throughput screening and the emergence of conditioned media and tissue-derived organoids that have greatly reduced the time and cost of cultivation, GIT organoids are invaluable tools for predicting preclinical drug toxicity and screening for clinical therapeutic agents.

Although GIT organoids offer advantages for basic research and clinical applications, they still have limitations, including differences in drug exposure compared to physiological conditions, the absence of an immune system, insufficient precision in responses to modulators, difficulties in replicating the gastrointestinal tumor microenvironment, and ethical issues associated with tissue collection. First, drug exposure in organoids differs from *in vivo* GIT administration; typically, drug exposure occurs on the basolateral side. Using microfluidic devices or more precise drug delivery systems in experiments to ensure that drugs are exposed accurately to the organoids from the apical side can address this issue. Second, organoid models contain cell types that are limited to intestinal epithelial cells, restricting studies on immune responses and the effects of drugs on immune cells within organoids. Technologies for incorporating immune system components into organoid systems are still underdeveloped, require further research. Furthermore, GIT organoid responses to modulators exhibit limited precision, especially in small differences, which requires us to enhance the precision of experimental design, such as establishing uniform standards for organoid culture and experimental procedures, in order to reduce variability between experiments. Additionally, GIT organoids cannot fully replicate the GIT tumor microenvironment or achieve purification of GIT tumor organoids. To address this challenge, we can reconstruct the extracellular matrix using biomaterials and specific extracellular matrix components, regulate key biochemical factors, and perform gene editing and epigenetic modifications. These actions are intended to maximize the simulation of the extracellular matrix of tumor cells and genetic alterations in cancer. At the same time, by employing methods such as flow cytometry, immunomagnetic bead sorting, fluorescent protein labeling, and microfluidic technology, we can achieve the spatial separation and purification of tumor cells, enhancing the purity of organoid tumor cells. Finally, generating healthy organoids from the same individual for tissue or donor specificity studies poses ethical challenges due to the need for donor to undergo nonessential surgical procedures. Therefore, ethical review and informed consent are very necessary.

Despite the limitations of GIT organoids, a growing number of studies have confirmed their potential for personalized treatment of GIT diseases. Studies combining microvascularized intestinal organoids and GIT organoids with gene editing technologies have provided more accurate models for GIT disease research. Large-scale cancer organoid biobanks have already been

established, and to further improve precision medicine for GIT diseases, GIT organoid biobanks should be established to allow for faster preclinical studies of drugs and provide personalized medication guidance for patients with GIT diseases.

Author contributions

LZ: Writing–review and editing, Writing–original draft. DL: Writing–original draft, Writing–review and editing, Data curation. WL: Writing–review and editing, Methodology. JH: Writing–review and editing, Formal Analysis. MZ: Writing–review and editing, Data curation, Visualization. XL: Writing–review and editing, Investigation, Resources. TS: Writing–review and editing, Conceptualization, Supervision. ZJ: Writing–review and editing, Funding acquisition, Validation. JZ: Writing–review and editing, Methodology, Supervision. YW: Writing–review and editing, Funding acquisition, Software, Visualization.

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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.

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Recent advances in extracellular matrix manipulation for kidney organoid research

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The kidney plays a crucial role in maintaining the body's microenvironment homeostasis. However, current treatment options and therapeutic agents for chronic kidney disease (CKD) are limited. Fortunately, the advent of kidney organoids has introduced a novel *in vitro* model for studying kidney diseases and drug screening. Despite significant efforts has been leveraged to mimic the spatial-temporal dynamics of fetal renal development in various types of kidney organoids, there is still a discrepancy in cell types and maturity compared to native kidney tissue. The extracellular matrix (ECM) plays a crucial role in regulating cellular signaling, which ultimately affects cell fate decision. As a result, ECM can refine the microenvironment of organoids, promoting their efficient differentiation and maturation. This review examines the existing techniques for culturing kidney organoids, evaluates the strengths and weaknesses of various types of kidney organoids, and assesses the advancements and limitations associated with the utilization of the ECM in kidney organoid culture. Additionally, it presents a discussion on constructing specific physiological and pathological microenvironments using decellularized extracellular matrix during certain developmental stages or disease occurrences, aiding the development of kidney organoids and disease models.

KEYWORDS

extracellular matrix, kidney organoids, decellularized extracellular matrix, microenvironment, disease models

1 Introduction

The kidney, is a vital organ that plays an important role in maintaining the homeostasis of human body. Chronic kidney disease (CKD), a progressive and irreversible loss of kidney function, is becoming increasingly prevalent due to rising comorbidities such as diabetes, hypertension, obesity, and an aging population (Kishi et al., 2024; GBD Chronic Kidney Disease Collaboration, 2020). Current therapeutic approaches for CKD are limited, relying predominantly on antihypertensive agents, antidiabetic medications, and pharmacological strategies aimed at controlling disease progression (Kishi et al., 2024). These treatments, required prolonged administration and exhibited only moderate efficacy, failing to halt the progression of kidney injury to end-stage kidney disease (ESKD), defined as an eGFR below 15 mL/min/1.73 m^2 (Levey et al., 2005). Animal disease models has substantially enhanced our understanding of the CKD pathophysiology and the clinical pharmacodynamics (Schnell et al., 2022; Fu et al., 2024). However, the interspecies differences significantly

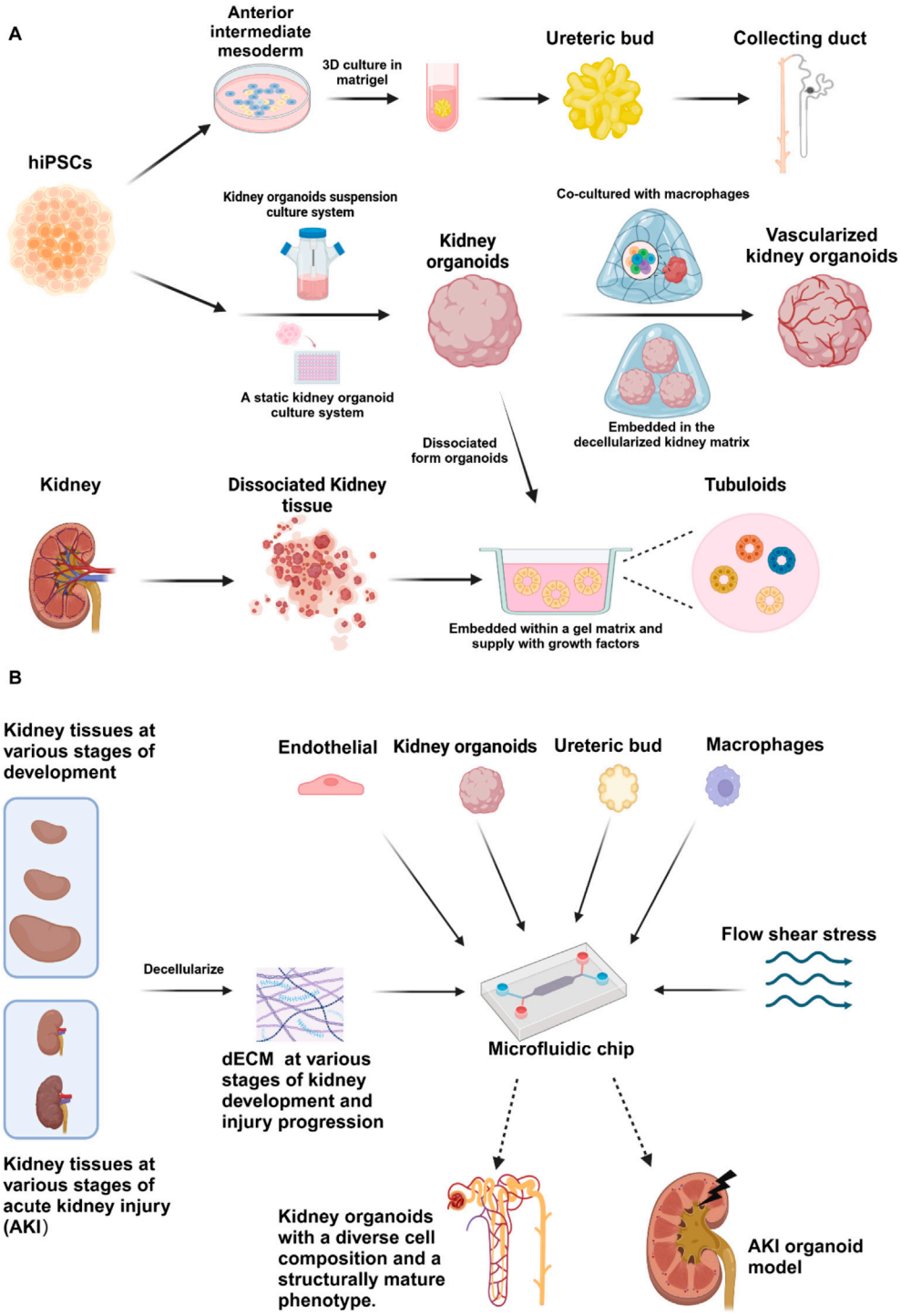

FIGURE 1
Various strategies are employed in the fabrication of kidney organoids (Created in BioRender.com). **(A)** Current procedures employed to construct ureteric bud (UB) organoids, kidney organoids and tubuloids using iPSCs and primary cells obtained from renal tissues. UB organoids are developed by differentiating hiPSCs into anterior intermediate mesoderm, which is subsequently embedded in Matrigel for 3D culture, leading to the formation of UB organoids that further differentiate into organoids containing collecting ducts. Kidney organoids, on the other hand, can be generated from hiPSCs using either suspension or static culture systems, with variations in the cultivation process resulting in vascularized kidney organoids. Finally, tubuloids are derived by embedding cells within a gel matrix and supplementing them with growth factors; their cellular origin may be either primary renal cells or dissociated organoids. Solid arrows indicate the processing steps, while dashed lines signify areas that have been magnified for clarity. **(B)** Schematic representation of further advancements in generation of sophisticated kidney organoids and acute kidney injury (AKI) organoid model achieved on organoids-on-chip system, incorporating the induction of decellularized renal extracellular matrix from specific developmental and pathological stages, co-culture with various exogenous cells, and introduction with fluidic shear stress, in order to simulate *in vivo* microenvironment. Solid arrows indicate the processing steps, while dashed arrows represent abstract model diagrams.

hinder the accurate extrapolation of disease mechanisms and the therapeutic efficacy. This challenge emphasizes the necessity for improved disease models that accurately reflect renal pathogenesis and facilitate precise drug screening approaches (Musah et al., 2024).

Organoids, as self-assembled 3D cellular structures *in vitro*, retain key characteristics of their *in vivo* counterparts and have emerged as powerful tools for developmental biology and drug screening. Kidney organoids have been a subject of research for nearly a decade (Taguchi et al., 2014). Over this time, their maturation has steadily advanced, enabling their use in constructing kidney disease models and drug screening (Musah et al., 2024; Tabibzadeh and Morizane, 2024; Dilmen et al., 2024; Long et al., 2024; Oishi et al., 2024; Chambers et al., 2023). However, their functional maturation and structural organization remain a challenge, in part due to the complexity of the kidney microenvironment (Garreta et al., 2019). The integration of extracellular matrix (ECM) components into organoid cultures has emerged as a promising strategy to mimic the *in vivo* environment, supporting more accurate tissue development and improving the functionality of kidney organoids (Kim J. W et al., 2022; Lacueva-Aparicio et al., 2022).

This article provides a comprehensive review of the current methods for the cultivation of kidney organoids (Figure 1A), with a particular focus on discussing the advantages and disadvantages of utilizing ECM for the culture of kidney organoids. It also proposes corresponding improvement strategies and outlines the future directions for the cultivation and application of kidney organoids.

2 Strategies for the construction of kidney organoids

Based on the single-cell sequencing data, 25 distinct cell types have been identified within the adult kidney tissue (Balzer et al., 2022). The development of the mammalian kidney initiates with the emergence of the nephrogenic cord, which is sequentially exposed to Wnt/β-catenin and BMP signaling to form the intermediate mesoderm (IM) (Schnell et al., 2022). This process provides two sources of progenitor cells for the differentiation of the collecting duct (CD) and the functional kidney units. Specifically, it involves the ureteric bud (UB), which originates from the anterior intermediate mesoderm (aIM), and the metanephric mesenchyme, which arises from the posterior intermediate mesoderm (pIM). However, currently kidney organoid culture strategies are unable to simultaneously provide appropriate culture conditions for both types of progenitor cells, which are requisite for replicating the reciprocal inductive signals observed *in vivo*. Specifically, GDNF secreted by MM initiates UB branching, while WNT9B secreted by UB initiates the mesenchymal-to-epithelial transition of the nephron (Oxburgh, 2018). Since the first report of nephron organoids induced from hiPSCs in 2014 (Taguchi et al., 2014), various protocols for constructing nephron organoids have endeavored to mimic the early kidney embryonic development. These protocols involved the induction of mesoderm formation in embryoid bodies through the activation of BMP4 and WNT signaling pathway using the GSK3 inhibitor (CHIR99021) (Lindsley et al., 2006; Magro-Lopez et al., 2024), followed by exposure to FGF9 to induce and maintain the niche of nephron progenitor cells (NPCs) (Muthukrishnan et al., 2015). The advancements in these methods have enabled the development of kidney organoids that provide a model that closely resembles human physiology, allowing the study of kidney biology at the organ level, and is superior to traditional two-dimensional culture systems or non-primate models.

Over the past decade, several laboratories have consistently improved the process of lineage reproduction of kidney organoids *in vitro*. Resulting in models that feature a broader spectrum of specialized cell types and increased structural complexity (Table 1). These advancements have proven to be crucial in understanding the pathogenesis of human kidney diseases and facilitating extensive drug screening (Taguchi and Nishinakamura, 2017; Takasato et al., 2014; Takasato et al., 2015; Kumar et al., 2019; Freedman et al., 2015; Huang et al., 2024; Li et al., 2016; Low et al., 2019; Morizane and Bonventre, 2017; Lawlor et al., 2021) (Table 1). However, the current established kidney organoids are characterized by an immature fetal state and transcriptionally similar to the first or second trimester of human fetal kidney, and lack an integrated vascular system, severely limiting their growth rate and long-term culture *in vitro*. A multimodal atlas of kidney organoid differentiation has delineated at least 15 highly specialized cell types, with off-target cell proportions varying from 6% (Combes et al., 2019) to 20% (Wu et al., 2018). Notably, the reproduction of distal cell types (mainly distal tubule and collecting duct cells) in organoids is comparatively less sophisticated than that of proximal cell types (mainly proximal tubule cells) (Yoshimura et al., 2023). Consequently, although kidney organoids demonstrate morphological similarity to the developing renal tissue, they encounter significant hurdles in attaining complete maturation and intricacy, especially with regard to replicating the *in vivo* filtration capabilities.

Furthermore, attempts have been dedicated to construct higher-order kidney organoids with higher lineage integrity and fully recapitulating *in vivo* renal developmental structures. To achieve this, some groups have developed ureteric bud (UB)/CD organoids derived from hiPSCs or UB progenitor cells extracted from mouse and human fetal kidneys, characterized by expandable, serially passaged and repeat branching morphogenesis (Howden et al., 2021; Mae et al., 2020; Shi et al., 2023; Uchimura et al., 2020; Zeng et al., 2021). Additionally, several proof-of-concept studies generated engineered kidney by aggregating 3D co-cultured NPCs with UB organoids derived from mice (Zeng et al., 2021), which preliminarily replicated the interconnected nephron and CD structures mimicking the reiterative inductive process of kidney development *in vitro*, shedding light on the developmental and regeneration mechanisms of the CD system (Zeng et al., 2021). However, akin to the limitations of nephron organoids, CD organoids derived from UB progenitors remain phenotypically immature compared to their *in vivo* counterparts, and functional evaluation demonstrating secretion and electrolyte reabsorption process is yet to be fully established (Mae et al., 2020).

To overcome the limitations of the extensive induction time and inadequate maturity of kidney organoids, researchers have turned to the induction and cultivation of tubuloids derived from primary renal tubular epithelial cells which were also found in urine (Gijzen et al., 2021; Schutgens et al., 2019). Intriguingly, tubuloid-derived cells can form polarized, leak-tight kidney tubules capable of

TABLE 1 Induction strategies for kidney organoids.

Ref.	Sources	Improvement	Method	Organoids
Krupa et al., 2024 (PMID: 38984433)	hiPSCs	Support the growth and maturation of kidney organoids	Cultured in Self-assembling polypeptide hydrogels and GelMA hydrogels	Kidney organoids
Garreta et al. (2019) (PMID: 30778227)	hiPSCs	Accelerate the differentiation of kidney organoids	Cultured in 1kp polyacrylamide hydrogel	
Homan et al., 2019 (PMID: 30742039)	hiPSCs	Generates vascularized kidney organoids with more mature podocyte, enhanced cellular polarity	3D-printed millifluidic chips were embedded in gelatin-fibrin ECM and applied for low or high fluid shear stress	
Sun et al. (2020) (PMID:32698872)	hiPSCs	The usc organoids self-organized well with no significant cell death	Cultured in optimal kidney ECM	Usc organoids
Lee et al., 2021 (PMID:34748091)	hiPSCs	More mature podocytes and vascular structures	Microfluidic chip was coated with 1.5% Matrigel and 1.5% Matrigel containing 100 ng/mL VEGF	Kidney organoids
Kim J. W et al. (2022) (PMID: 35322595)	hiPSCs	Increase the formation of blood vessel network and promote the maturation of kidney organoids	Kidney decellularized matrix hydrogel	
Nerger et al., 2024 (PMID: 38180232)	hiPSCs	Affect the roundness of nephron segments, spatial localization and the ratio of glomerulus to tubules	Sodium alginate hydrogel	
Pecksen et al. (2024) (PMID: 38702808)	hiPSCs	Decrease the apoptosis of iPSCs induced by CHIR, promote the differentiation of renal organoids and promote vascularization	A monocyte of human origin coculture with iPSCs by transwell cuture system	
Garreta et al. (2024) (PMID: 38762768)	hiPSCs	Maintain organoid differentiation and promote vascularization	Kidney derived decellularized matrix hydrogel, hPSCs derived endothelial organoids were embedded for three-dimensional culture	
Maggiore et al. (2024) (PMID: 38901605)	hiPSCs	Increase the vascularization of renal organoids and improve the maturity of nephron. Generate different endothelial cell subtypes	iETV2-iPSCs were integrated into the previously constructed renal organoid system, and ETV2 expression was induced at day5 after culture	
Takasato et al. (2015) (PMID: 26444236)	hiPSCs	The first report for *in vitro* kidney organoids induction; Optimization the exposure period of Wnt, FGF, and RA in fate selection	2D induced cell were spun down to form a pellet and transferred onto a Transwell under the sequential induction by Wnt, FGF	
Freedman et al. (2015) (PMID: 26493500)	hiPSCs	Simplified induction procedure; validate the Genome-modified kidney organoids form PKD-specific cysts	Induction Procedure followed by the formation of cavitated spheroids, then through MET induction to form nephron-like organoids	
Morizane et al. (2015) (PMID: 26458176)	hiPSCs	First reported strategy to replated 2D NPCs into 3D suspension culture with distinct lumens mimics the nephron	Generation of SIX2+SALL1+WT1+PAX2+ NPCs with high efficiency followed by the formation of PAX8+LHX1+ renal vesicles that self-organized into 3D nephron structures	
Taguchi and Nishinakamura (2017) (PMID:29129523)	Mouse and hiPSCs	Higher-order structures with branching morphogenesis by co-culturing independently induced ureteric bud, nephron and stromal progenitor lineages	Identified mutually distinct inductive signals between the NP and UB lineages in every step of differentiation; Co-culture iNPs, iUB, and the Pdgfra+ SP population sorted from E11.5 embryonic kidneys. iUB: induced UB; iNPs: NP induction from the mESCs; SP: stromal progenitors	
Li et al. (2016), Huang et al. (2024) (PMID:27570066 PMID:38692273)	Primary mouse and human NPCs/hiPSCs	Develop systems for long-term expansion of induced NPCs generated nephron organoids with minimal off-target cell types and enhanced maturation of podocytes relative to other strategies; podocyte reprogramming to an NPC-like state	Manipulation of p38 and YAP activity allowed for long-term clonal expansion of primary mouse and human NPCs and induced NPCs from hiPSCs	
Vanslambrouck et al., 2022, 2023 (PMID:37770563 PMID:36209212)	hiPSCs	PT-enhanced organoids with distinct S1 - S3 proximal tubule cell types; improved albumin and organic cation uptake, improved expression of SARS-	Extended the monolayer differentiation of nephron progenitor to 12–14 days cultured in enhanced BMP7 condition to 10 ng/mL	

(Continued on following page)

TABLE 1 (*Continued*) Induction strategies for kidney organoids.

Ref.	Sources	Improvement	Method	Organoids
		CoV-2 entry factors resulting in increased viral replication		
Przepiorski et al. (2018), Przepiorski et al. (2021) (PMID: 30033089 PMID:33938892)	hiPSCs	Establish a fast, efficient and cost-effective suspension culture method, allows large-scale organoid production	Bioreactor-Based	
Kumar et al. (2019) (PMID: 30846463)	hiPSCs	Modified suspension culture method, a three- to fourfold increase in final cell yield and a 75% reduction in cost per million organoid-derived kidney cells compared with static culture	Low speed (60 rpm) swirling on an orbital shaker to form cell aggregates	
Lawlor et al. (2021) (PMID: 33230326)	hiPSCs	Rapid and high-throughput; highly uniformly patterned; increasing nephron yield	3D bioprinting	
Howden et al. (2021) (PMID: 33378647)	Distal nephron epithelim from kidney organoids	Shift the identity of this GATA3+/EPCAM + epithelial population toward UE, including ureteric tips, cortical and medullary UE, by altering the *in vitro* culture conditions	GATA3+/EPCAM + epithelial population isolated by FASC and induced in the presence of GDNF, CHIR, FGF2, ATRA and Y-27632	UB/Collecting duct organoids
Mae et al. (2020) (PMID:32726627)	hiPSCs	Induced UB organoids have tubular lumens and repeat branching and differentiated into collecting duct progenitors.	iPSC induced to AIM and then ND stage embedded in 2% Matrigel for 6 days to constitute induced UB organoids with epithelial polarity and tubular lumens	
Uchimura et al. (2020) (PMID: 33326782)	hiPSCs	Combine independently differentiated MM-like and UB-like progenitors to generate human kidney organoids with a collecting system. Detect for the first time urothelial (Uro) cells	Aldosterone and AVP drive collecting duct maturation	
Zeng et al. (2021) (PMID:34131121)	Mouse and human fetal kidneys; hiPSCs	UB organoids generate collecting duct organoids, with differentiated principal and intercalated cells Develope a screen to establish conditions supporting the differentiation of CD organoids	Sorting of KIT + cells were used to enrich the precursor population then induced in chemically-defined culture conditions	
Shi et al., 2023 (PMID:36038632)	hiPSCs	Exhibit authentic morphological behavior and responses to developmental stimuli; recapitulate the morphogenetic pattern in isolated UBs	Ensure at least 90% efficiency at the mesendodermal and pronephric intermediate mesoderm (IM) stages without using cell sorting or mechanical dissection	
Yousef Yengej et al. (2023) (PMID: 36724260)	iPSC-derived organoids	Selectively expand the mature functional renal epithelium without off-target cells and provide easy apical access that enables evaluation of tubular transport	Tubular fragments and cells from D7+18 organoids resuspended in Basement Membrane Extract (BME) gel and plated on suspension culture wells plates	Tubuloid organoids
Lindoso et al., 2022 (PMID: 35841001)	Tubuloid-derived cells + EVs	EVs from kidney tubular epithelial cells can phenotypically improve *in vitro* tubuloid maturation	Tubuloids cultured with EV	
Schutgens et al. (2019), Gijzen et al. (2021) (PMID:30833775 PMID: 33674788)	Adult kidney tissue or urine	Long-term growth and can be expanded for at least 20 passages; Model infectious, malignant and hereditary kidney diseases; Adopt a tubular conformation and display active (trans-) epithelial transport function	Establishing kidney tubuloids and characterization of tubuloid cell–derived 3D tubular structures in a perfused microfluidic multi-chip platform, the OrganoPlate 3-Lane	

Ref. for Reference, hiPSCs, for human indued pluripotent stem cells; USCs, for urine-derived stem cells, Extracellular vesicle (EVs), Ureteric bud (UB), Nephron progenitor cells (NPCs).

performing trans-epithelial transporter activity (Yousef Yengej et al., 2023). Tubuloids serve as a highly physiologically relevant model for simulating infectious, malignant, and genetic kidney diseases, including tubulopathies such as Fanconi syndrome (Jamalpoor et al., 2021), re-invigorating the understanding of renal transport mechanisms, drug screening, and personalized medicine. Nonetheless, the capacity of tubuloids to accurately replicate diseases with complex multi-cellular

interactions and intricate pathological mechanisms necessitates further investigation.

Although kidney organoid with similar degrees of differentiation have been established via cohort of procedures, most induction programs are time intensive and require the administration of costly exogenous growth factors, severely limiting the development of large-scale organoid culture strategies (Morizane and Bonventre, 2017). The complexity and high costs associated with these methods pose significant barriers to their widespread use in research and therapeutic applications. Therefore, establishing a controllable, and highly reproducible culture process, is essential for optimizing the lifespan, architecture complexity, homogeneity, and differentiation fidelity of organoids. This prospect is crucial for the standardization and large-scale generation of the next-generation organoids. Alan's (Przepiorski et al., 2018; Przepiorski et al., 2021) and Little's laboratory (Kumar et al., 2019) have developed suspension organoid culture systems resulted in a 3-4 fold increase in final cell yield compared to static culture, providing a highly promising platform for the automation and large-scale production of kidney organoids. Furthermore, the integration of bioprinting technologies can automate the production of organoids with highly homogeneity in conformation, improving the throughput of manufacture up to 9 fold (Lawlor et al., 2021). The large-scale production of kidney organoids via bioengineering strategies provides versatile platform for optimizing organoid technology towards revolutionizing the regenerative medicine and clinical applications. The integration of automated high-throughput imaging techniques enables the phenotypic analysis of kidney organoids, which can be utilized for drug screening related to nephrological disorders (Czerniecki et al., 2018; Wang et al., 2022).

In summary, the advancements of kidney organoids with the capability of recapitulating early temporal-spatial embryonic developmental trajectories are now used as faithful substitutes in studying kidney development *in vitro*. Organoid models enable the reproduction of tissue structures, providing opportunities to investigate the mechanism of kidney development and disease through functional screening (Huang et al., 2024). However, the efficacy of kidney organoid models depends on their developmental fidelity to primary tissue, and to what extent can they mimic embryonic organ development on cellular characteristics and architectural complexity levels remains challenging (Little and Combes, 2019). Consequently, modifying the *in vivo* biophysical microenvironment in spatiotemporal dimensions exhibits great potential for driving the determination of cell fate and commitment to lineage during organoid development.

3 The construction of a microenvironment specific to kidney tissue facilitates the differentiation of kidney organoids

Early embryonic development involves the formation of three germ layers, where cell fate is regulated by intracellular and extracellular signaling pathways. A multitude of studies have underscored the determinant role of specific transcription factors in lineage commitment (Wang et al., 2013; Takahashi and Yamanaka, 2006; Rigillo et al., 2021; Chen et al., 2024). However, research on how to specifically regulate cell fate through extrinsic signals is still limited (Walma and Yamada, 2020). The ECM significantly influences cell fate by activating various signaling pathways, which play a crucial role in cell fate decisions (Tang et al., 2022; Tang et al., 2013; Amran et al., 2024; Kersey et al., 2024; Li et al., 2024; Zhang et al., 2023; Sun et al., 2020). Therefore, elucidating the composition and dynamic changes of the ECM during the processes of cell development, aging, and disease progression is crucial for simulating and constructing the microenvironment of tissue at different developmental stages, injuries, and pathological processes.

The complexity of the ECM arises from its diverse constituents, including core structural proteins and regulatory factors that can initiate ECM remodeling and impact development and disease (Yamada et al., 2022; Rekad et al., 2022; Zhou et al., 2018; Damjanovski et al., 2001; Kaneko et al., 2024). Advances in tissue engineering allow for the simulation of ECM using biomaterials to achieve *in vitro*/*in vivo* cell fate regulation. However, disparities exist between commercial biomaterials and tissue ECM, which influence cell fate regulation and the efficacy of disease treatment (Zhang et al., 2023; Kim S. et al., 2022). Decellularized extracellular matrix (dECM) hydrogels are prepared through chemical or physical decellularization processes that remove immunogenic and pathogenic elements from natural tissues, followed by freeze-drying, grinding, and enzymatic digestion. These hydrogels retain the majority of bioactive proteins from the original tissue (Zhang W. et al., 2021). Therefore, the use of dECM derived from tissues to simulate the physiological microenvironment has attracted increasing attention for organoid studies. For example, Sun et al. demonstrated that the dECM hydrogels from spinal cord of neonatal rabbits can promote the axonal growth and functional maturation of spinal cord organoids (Sun et al., 2024). Similarly, in the aging process, the composition and mechanical properties of the ECM have also changed, thereby affecting tissue function. Culturing normal human mammary epithelial cells with the ECM from aged breast tissue reinforced the invasive capability of cells, and increased the expression of inflammatory cytokines and cancer-related genes and proteins (Bahcecioglu et al., 2021). Moreover, cervical squamous cell carcinoma (CSCC) patients' adjacent cervical tissue can be used to prepare uterine cervical extracellular matrix (UCEM) hydrogels, which faithfully defined the microenvironment of cervical cancer tissue. CSCC organoids cultured with UCEM hydrogel exhibit superior characteristics compared to those cultured with Matrigel, as evidenced by increased expression of cervical cancer-related genes and signaling pathways, resulting in a closer resemblance to patient-derived CSCC tissues (Song et al., 2024). The above studies indicated that the preparation of dECM from tissues under different physiological/pathological conditions can help construct more mature organoids and disease models.

The influence of extracellular matrices (ECMs) on renal development and functionality has been extensively investigated, yielding insights into various aspects such as kidney morphogenesis, branching patterns, pathologies, and regenerative processes (Abdollahzadeh et al., 2022). Several research groups have employed proteomics to analyze the ECM composition in normally developing kidneys, aging kidneys, and kidney diseases (Diedrich et al., 2024; Rende et al., 2023; Randles et al., 2021; Eckersley et al., 2023; Li et al., 2023; Lipp et al., 2021; Lennon

et al., 2014). Understanding the composition and dynamic changes of kidney ECM under different physiological and pathological conditions provides the basis for constructing microenvironments of renal tissues with diverse physiological and pathological characteristics. Furthermore, a series of studies has utilized kidney dECM for renal cell culture (Quinteira et al., 2024; Bongolan et al., 2022; Sobreiro-Almeida et al., 2020), renal injury repair (Kim et al., 2024), and organoid culture (Kim J. W et al., 2022; Garreta et al., 2024). In terms of renal cell culture, dECM-based hydrogels have been shown to effectively support renal progenitor cell survival, proliferation, and differentiation into tubular cells and podocytes, thereby providing a biocompatible platform conducive to renal regeneration (Quinteira et al., 2024). Furthermore, optimizing the decellularization process—such as using lower concentrations of SDS during the procedure—helps to preserve essential ECM components, enhancing renal cell survival and distribution, although challenges remain regarding mature cell migration (Bongolan et al., 2022). Additionally, dECM can serve as a substitute for the tubular basement membrane, simulating the physiological relevance of the *in vivo* environment. Co-culturing renal progenitors with endothelial cells has enabled the construction of a tubular bilayer model, which mimics the native tissue environment more closely (Sobreiro-Almeida et al., 2020). In the aspect of renal injury repair, an implantable decellularized extracellular matrix sponge has demonstrated not only rapid hemostasis during partial nephrectomy surgery but also superior wound healing, offering a promising solution for both managing renal hemorrhage and enhancing tissue regeneration at the lesion site (Kim et al., 2024). Collectively, these studies highlight the potential of dECM to advance renal research and therapeutic applications, including the enhancement of renal cell cultures and injury repair. Decellularized materials created in various laboratories have demonstrated the ability to promote differentiation, maturation, vascularization, and the development of tubular and glomerular-like structures in kidney organoids (Kim J. W et al., 2022; Garreta et al., 2024), reinforcing the promising role of dECM in advancing both basic research and clinical applications. Additionally, some laboratories have developed decellularized matrices from fibrotic kidneys to assess the impact of dECM on endothelial progenitor cells (Zhang R. et al., 2021). Although studies have not yet reported how these dECMs derived from pathological kidneys impact kidney organoid differentiation, they hold potential for constructing disease model organoids that may better simulate pathological conditions. However, there are still gaps in the maturity of kidney organoids (including the presence of precursor cells and cell cycle cells), the representation of cell types (lacking pericytes and distal tubular cells), and structural complexity (vascular wrapping and podocyte wrapping structures) compared to mature renal tissues (Kim J. W et al., 2022). In addition, the kidney organoids may contain off-target cell populations (Kim J. W et al., 2022). One potential explanation is that the current manufactured dECM primarily recapitulates the matrue renal-favor microenvironment. In contrast, kidney organoids are usually generated from hiPSCs, which contain numerous cells in the early stages of differentiation. As a result, the dECM derived from mature tissues may not be optimal for supporting the maturation of these early-stage differentiated cells in kidney organoids, leading to hindrances in their development. This mismatch between dECM derived from mature tissue and kidney organoids composed of early-stage differentiated cells highlights a crucial challenge in the field. Despite the absence of direct studies comparing early-stage and mature kidney dECM in renal organoid cultures, clues can be drawn from existing studies on the dECM in other organ systems. For instance, a study on rabbit spinal cord dECM found that neonatal dECM contained higher levels of proteins like pleiotrophin (PTN) and tenascin (TNC), which promote neural development, axonal growth, and regeneration, while mature dECM had more inhibitory components like chondroitin sulfate proteoglycans (CSPGs), limiting regenerative potential (Sun et al., 2024). This shift in ECM composition highlights a potential mismatch when applying mature tissue-derived ECM to support the maturation of progenitor cells in organoids. Early-stage ECM is optimized for promoting cell proliferation and differentiation, while mature ECM may lack these developmental cues, potentially hindering organoid maturation and limiting its functionality. By understanding and mimicking the developmental ECM environment, researchers may be able to better support the maturation and functional development of organoids, leading to more effective tissue models for both research and therapeutic applications.

4 Conclusion and prospect

Amidst the rapid advancements in multidisciplinary technologies, despite significant advancements in cellular diversity, structural complexity, functional repertoire, and developmental maturity of kidney organoids, a discernible disparity remains when compared to mature renal tissues. To address this, one potential method is the construction of a tissue microenvironment based on tissue-specific dECM, which could facilitate the maturation of kidney organoids. Both human and porcine renal dECM have been found to promote the differentiation of kidney organoids (Garreta et al., 2024). This discovery not only paves the way for potential commercialization of renal dECM but also addresses ethical concerns related to the use of human dECM.

Although current dECM derived from mature renal tissues can partially promote the maturation and vascularization of organoids, there are limitations in terms of cell types and structures, with the presence of non-renal cell types. The continued differentiation and maturation of kidney organoids require an ECM that is distinct from mature renal tissues. To address this, single-cell sequencing can be utilized to analyze various stages of kidney development and aging, as well as different regions. Furthermore, the ECM can be identified using mass spectrometry. Through the integration and comparison of single-cell multi-omics data at different development stages of kidney and kidney organoid differentiation, it becomes possible to identify the ECM that best corresponds to the kidney organoids. Culturing organoids with the corresponding stage's ECM and introducing exogenous cells such as macrophages (Liu et al., 2020; Pecksen et al., 2024) and endothelial cells (Maggiore et al., 2024), a complex cellular microenvironment can be constructed to simulate physiological conditions to the greatest extent (Figure 1B). Furthermore, microfluidic chips can be utilized to apply fluid shear stress to the three-dimensional co-cultured organoids, thereby mimicking the processes of kidney development, aging, and

disease (Figure 1B). Developing organoids at these specific stages can help elucidate the mechanisms of development, aging, and disease occurrence, and also provide a promising direction for drug screening in nephropathy using kidney organoids.

There are numerous causes of kidney disease, including congenital genetic conditions such as polycystic kidney disease (Cornec-Le Gall et al., 2018), as well as a significant proportion of kidney diseases induced by nongenetic factors, such as obstructive nephropathy or nephrotoxic drugs leading to acute kidney injury (AKI) (Chávez-Iñiguez et al., 2020; Perazella and Rosner, 2022). Genetic factors, which induced kidney diseases can be modeled by gene editing of hiPSCs followed by the induction of kidney organoid to obtain the corresponding disease models. However, there is still limited research on how to construct kidney organoid disease models induced by nongenetic factors. Although several organoids models of AKI have been developed through the use of various inflammatory stimuli or nephrotoxic drugs (Morizane et al., 2015), there is still a certain gap between these organoid models and AKI due to the maturity of organoids (Bejoy et al., 2022). Moreover, due to the multitude of causes of AKI, various alterations in ECM proteins are also markers of AKI, such as nidogen-1 glycoprotein (Gui et al., 2024) and Metalloproteinase 1 and 3 (Klimm et al., 2024). However, our understanding of the dynamics of the overall ECM composition and cellular microenvironment during the occurrence and development of AKI is still limited. Therefore, how to use the ECM related to AKI diseases in combination with kidney organoids to construct a more physiologically relevant AKI model is also a direction for future research.

In summary, kidney organoids serve as crucial multicellular models for studying renal development, aging, and disease *in vitro*, and offer distinct advantages over traditional animal and cell models. Their greatest strength lies in the presence of multiple interacting cell types and a certain level of physiological structure, allowing them to simulate the microenvironment of kidney tissue *in vitro*. However, there remains a gap between current kidney organoids and mature renal tissues, both in terms of cell types and maturity. Furthermore, there is limited research on constructing organoids that precisely mimic specific stages of human kidney development, aging, and disease. One viable approach to address these challenges involves utilizing kidney dECM that correspond to the developmental stages of tissue. By co-culturing immune-related cells and creating a complex cellular microenvironment that closely resembles physiological conditions, it becomes possible to obtain more differentiated cell types and maturity in kidney organoids. Subsequently, these advanced models enable more accurate and reliable drug screening. Furthermore, the application of microfluidic chip technology enables the construction of micro-physiological models that replicate multi-organ interactions in disease states, facilitating the study of organ interactions under normal physiological and disease conditions and also drug screening. These avenues represent future directions for the advancement of kidney organoid research.

Author contributions

RW: Writing–original draft, Writing–review and editing. YS: Writing–original draft, Writing–review and editing. QL: Writing–review and editing. YX: Writing–review and editing. SL: Writing–review and editing. WG: Writing–review and editing. YX: Conceptualization, Writing–review and editing. SZ: Conceptualization, Supervision, Writing–original draft, Writing–review and editing.

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Conflict of interest

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

Publisher's note

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Comprehensive analysis of IRF8-related genes and immune characteristics in lupus nephritis

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Background: There are currently no reliable diagnostic biomarkers or treatments for lupus nephritis (LN), a complication of systemic lupus erythematosus. Objective: We aimed to explore gene networks and potential biomarkers for LN by analyzing the GSE32591 and GSE113342 datasets from the Gene Expression Omnibus database, focusing on *IRF8* and *IRF8*-related genes.

Methods: We used differential expression analysis, functional enrichment, protein-protein interaction (PPI) network construction, and the CIBERSORT algorithm for immune infiltration assessment. To validate the expression levels of the IRF8 gene in the kidneys of lupus mice models, we used quantitative real-time PCR (qRT-PCR) and Western blotting (WB). A diagnostic classifier was built using the RandomForest method to evaluate the diagnostic potential of selected key genes. To bridge our findings with potential therapeutic implications, we used the drug-gene interaction database to predict drugs targeting the identified genes.

Results: Twenty co-differentially expressed genes (DEGs) were identified, with IRF8 exhibiting significant expression differences and potential as a biomarker. Functional enrichment analysis revealed pathways associated with immune response. Validation through qRT-PCR and WB confirmed that the IRF8 gene and its protein exhibited elevated expression levels in the kidneys of lupus mice compared to control groups. The diagnostic classifier revealed impressive accuracy in differentiating LN from control samples, achieving a notable area under the curve values across various datasets. Additionally, immune infiltration analysis indicated significant differences in the immune cell profiles between the LN and control groups.

Conclusion: IRF8 and its related genes show promise as biomarkers and therapeutic targets for LN. These findings contribute to a deeper understanding of the molecular mechanisms involved in LN and may support the development of precision medicine strategies for improved patient outcomes.

KEYWORDS

lupus nephritis, IRF8, biomarkers, drug-gene interaction, immune infiltration

1 Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease of unknown etiology, affecting approximately five million people worldwide (Kong et al., 2019; Udhaya Kumar et al., 2020). The female-to-male ratio among patients with SLE is approximately 7–9:1 (Kong et al., 2019). In patients with SLE, immune dysregulation leads to the production of autoantibodies targeting nuclear and cytoplasmic antigens. SLE also initiates autoimmune responses and inflammation across multiple organs, giving rise to a broad spectrum of clinical manifestations. Mild cases may be limited to skin involvement, such as erythema or oral ulcers, while severe cases can involve critical damage to the hematologic, renal, or nervous systems, potentially posing life-threatening risks. (Yap and Chan, 2019). LN is a manifestation of SLE that affects approximately 39% of patients (Kong et al., 2019) and is a major risk factor for morbidity and mortality (Almaani et al., 2017). LN is characterized by glomerulonephritis (Yang and Li, 2019). About 10% of all patients with LN develop end-stage renal disease (ESRD) (Almaani et al., 2017). However, the pathogenesis of LN remains unclear. As LN has complex clinical manifestations and no specific treatment, biomarkers and treatment targets are urgently needed. With the advancement of bioinformatics and metabolomics, increasing numbers of researchers are focusing on discovering biomarkers for the early diagnosis of LN. Due to their non-invasive nature, these methods may potentially serve as alternatives to renal biopsy. Beyond classical serum markers for LN, such as anti-double-stranded DNA (anti-dsDNA) antibodies and C1q, abnormal DNA methylation, non-coding RNA, and variations in levels of chemokines, interleukins, and urinary proteins may all serve as potential new biomarkers (Alduraibi and Tsokos, 2024). And the recent advent of gene testing and bioinformatics analysis has gradually elucidated associations between genes and diseases.

2 Materials and method

2.1 Data download

GSE32591 (Bethunaickan et al., 2011) and GSE113342 (Mejia-Vilet et al., 2019) are two sets of gene expression profile data for LN, downloaded from the official NCBI GEO website (https://www.ncbi.nlm.nih.gov/geo/) (Barrett et al., 2007) using the GEOquery package in R (Davis and Meltzer, 2007). GSE32591 and GSE113342 were divided into tubular interstitial (TUB) and glomerular (GLOM) gene expression groups. The gene expression profiles of patients with LN and patients in the control group are presented in Table 1.

TABLE 1 GEO database of LN patient data.

ID	GLOM (LN)	GLOM (LD)	TUB (LN)	*TUB* (LD)
GSE32591	32	14	32	15
GSE113342	28	6	28	10

TUB, tubular interstitial; GLOM, glomerular.

2.2 Co-differentially expressed genes (DEGs)

To investigate the effect of gene expression on patients from the LN and normal sample groups, data from GSE32591 and GSE113342 were divided into TUB and GLOM categories. The limma package in R was used to analyze the differences between groups (Ritchie et al., 2015). DEGs were determined using an adjusted p-value threshold of <0.05. Specifically, genes with logFC >1 and an adjusted p-value <0.05 were deemed upregulated DEGs, while those with logFC < −1 and an adjusted p-value <0.05 were categorized as downregulated DEGs. The DEGs identified across the four datasets were compared and intersected to identify the co-DEGs. These DEGs were visualized using the ggplot2 package in R (Maag, 2018). Additionally, the effects of shared DEGs on patient stratification were assessed using the heatmap package in R.

2.3 co-DEG function and pathway enrichment analysis

In extensive gene enrichment studies, gene ontology (GO) functional annotation analysis was used to explore biological processes (BP), molecular functions (MF), and cellular components (CC). The Kyoto encyclopedia of genes and genomes (KEGG) database (Kanehisa and Goto, 2000) served as a repository for information related to genomes, biological pathways, diseases, and drugs. The clusterProfiler package in R was used to perform GO function annotation and KEGG pathway enrichment analyses of the identified co-DEGs (Wu et al., 2021). A $p < 0.05$ was considered the threshold for statistical significance.

2.4 Protein-protein interaction (PPI) network construction

The STRING database, comprising established and predicted PPIs, was used to construct a PPI network (Szklarczyk et al., 2019). The PPI network model was visualized using Cytoscape software (Shannon et al., 2003). Local clusters within the network, characterized by closely connected interactions, could suggest molecular complexes associated with biological functions. Pearson's correlation coefficients were calculated between the expression levels of IRF8 and other genes to analyze the gene expression profile data from the four datasets, with statistical significance set at $p < 0.05$. Genes demonstrating a significant correlation with IRF8 in at least three datasets were identified as IRF8-associated genes. Selecting IRF8-associated genes was depicted using the VennDiagram package in R (Chen and Boutros, 2011). DAVID, an online resource located at http://david.abcc.ncifcrf.gov/, integrates biological datasets and analytical tools to facilitate the construction of extensive gene or protein lists, provides detailed annotations of biological functions, and supports the analysis of biological information about these lists. In this study, DAVID was utilized for function annotation and pathway enrichment analysis, considering a $p < 0.05$ to be statistically significant.

2.5 Diagnostic prediction, model construction, and verification

Random forest is a bagging-based ensemble learning method used for regression, classification, and other applications (Brieuc et al., 2018). It is highly accurate, rapidly trained, and easy to implement, and it also performs variable-importance ranking. We intersected IRF8-related genes with co-DEGs to identify key genes and further evaluated the impact of their expression levels on patient diagnosis. The random forest algorithm was used for classification and was implemented using the RandomForest package in WEKA. Feature selection was performed, and a diagnosis prediction model was built using the RandomForest package in R (Svetnik et al., 2003), resulting in a classifier for the feature genes. During the construction and validation of the classifier, the training set comprised GLOM samples from the initial analysis of GSE32591, which included 32 LN and 14 normal tissues, along with TUB samples from the same dataset and GLOM and TUB samples from the GSE113342 dataset. The classifier model was then tested, and the pROC package in R was used to generate ROC curves and compute the AUC (Robin et al., 2011).

2.6 Immune infiltration analysis

CIBERSORT, available at https://cibersort.stanford.edu/, utilizes linear support vector regression to deconvolute immune cell subtype expression from gene expression matrices based on predefined reference profiles and a set of gene expression features representing 22 white blood cell subtypes (Newman et al., 2019). In this study, RNA-Seq data were used to estimate the levels of immune cell infiltration. The CIBERSORT algorithm was subsequently employed to evaluate the relationship between the co-DEGs and immune cell infiltration.

2.7 Mice

Female MRL/Mpj and MRL/lpr mice were obtained from Shanghai Jihui Laboratory Animal Care Co. Ltd (Shanghai, China) and housed in a pathogen-free facility at Fudan University. The Institutional Animal Care and Use Committee of Fudan University approved all animal experiments.

2.8 Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA extraction from tissue samples was performed using TRIzol Reagent (15,596,026, Invitrogen, United States) following the manufacturer's guidelines. The PrimeScript RT Reagent Kit (Takara, Japan) was used for cDNA synthesis. Expression levels of IRF8 were quantified using TB Green Premix Ex Taq II (Takara, Japan) on a QuantStudio 6 Flex Real-Time PCR System (ABI, United States). Data were analyzed using the delta-delta Ct method. Primer sequences are provided in Supplementary Table S1.

2.9 Western blot

Samples were lysed using RIPA lysis buffer (P0013C, Beyotime, China). The protein concentrations were measured using a BCA assay kit (P0010S, Beyotime, China). Proteins were then analyzed through standard Western blotting techniques. Membranes were incubated overnight at 4°C with primary antibodies, specifically anti-interferon regulatory factor 8 (IRF-8) (1:1,000, 5,628, CST, United States) and GAPDH (1:1,000, 2,118, CST, United States). Following washing, the membranes were incubated with goat anti-rabbit IgG secondary antibody (1:2000, 7,074, CST, United States) for 1 h at room temperature. Detection was carried out using ECL reagent (Millipore, United States), and images were obtained using a LAS-3000 imager (Fujifilm, Japan). Image quantification was conducted using Photoshop (Adobe).

2.10 Regulatory networks and target drugs of hub genes

The potential influence of drugs on the expression of hub genes was investigated using the drug–gene interaction database (DGIdb), which aggregates drug-gene interaction information from 30 different sources (Cotto et al., 2018). Additionally, the Cytoscape software was employed to facilitate a more detailed analysis of the drug network, enhancing the examination of the interactions within (Shannon et al., 2003).

2.11 Statistical analysis

Data processing and analysis were conducted using the R software (version 4.0.2; R Core Team, Vienna, Austria). Graphical representations were generated using the ggplot2 package. The pROC package in R (Robin et al., 2011) was used to create ROC curves, compute the AUC, assess the accuracy of risk scores, and predict prognosis. Statistical comparisons between the two groups were conducted using the Student's t-test, with a $p < 0.05$ considered statistically significant.

3 Results

3.1 Co-DEGs

We used the limma package to perform differential expression analysis to investigate the impact of gene expression in patients with LN compared to normal controls. This allowed us to identify DEGs, categorized into upregulated and downregulated DEGs across four sample groups, as demonstrated in Figures 1A–D and Supplementary Table S2. We then intersected the downregulated DEGs from dataset GSE32591 with those from GSE113342 and the upregulated DEGs from GSE32591 with those from GSE113342. This process identified 20 co-DEGs. To assess the relevance of these co-DEGs for clinical diagnosis,

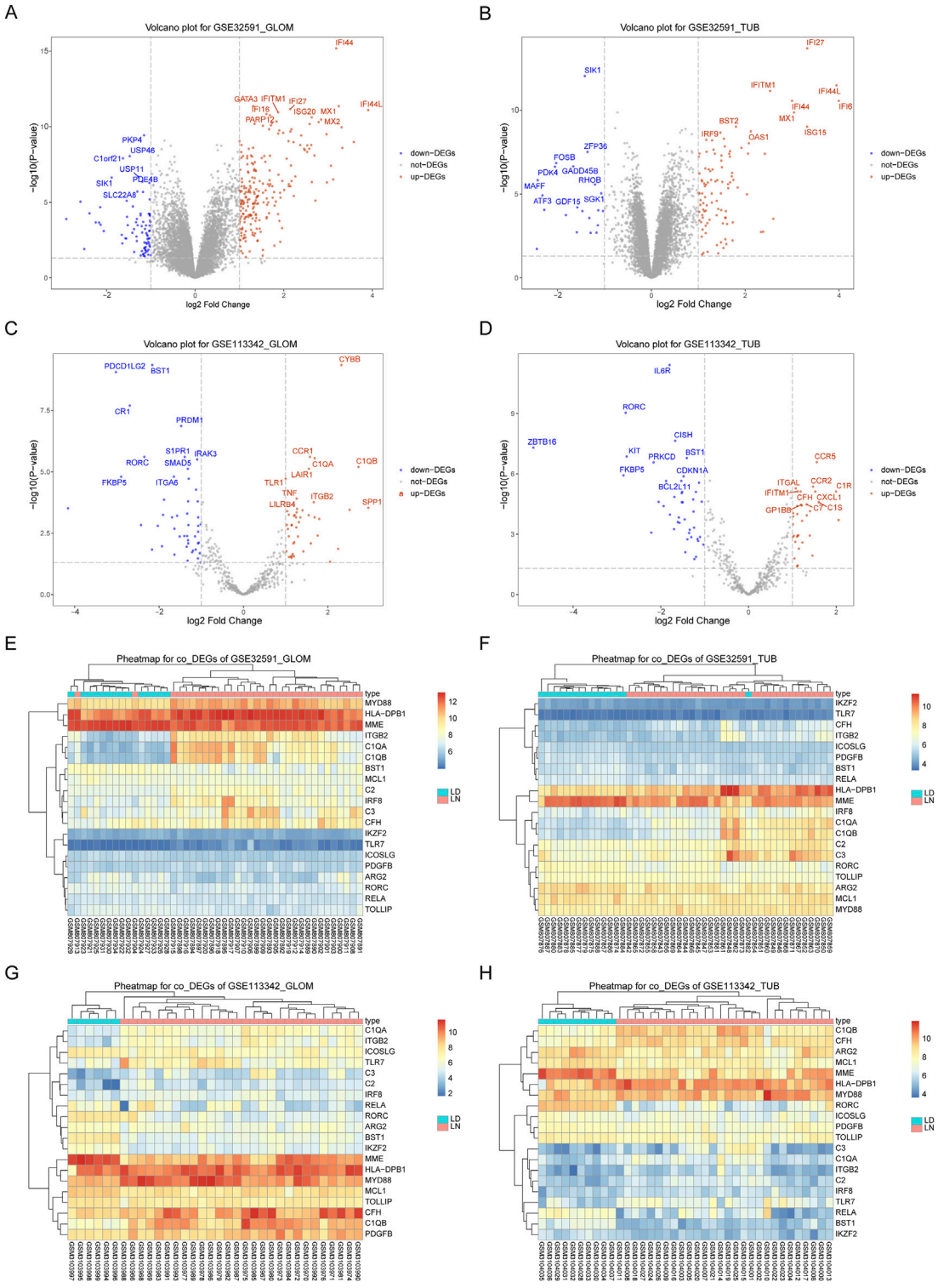

FIGURE 1
Differentially Expressed Genes in GSE32591 and GSE113342. **(A–D)** Volcano plots showing differentially expressed genes with log2(Fold Change) on the x-axis and -log10(p.adjust) on the y-axis. **(E–H)** Heatmaps showing expression levels of differentially expressed genes across patient samples.

classification heatmaps were generated (Figures 1E–H). The heatmaps demonstrated that the 20 co-DEGs differentiated the disease samples from the normal ones. Furthermore, statistical analysis of the gene expression levels within the co-DEGs indicated a significant elevation in IRF8 expression in the LN group (Figure 2).

3.2 Functional enrichment analysis of co-DEGs

To explore the relationship between co-DEGs and various BP, MF, CC, and pathways, we performed functional enrichment

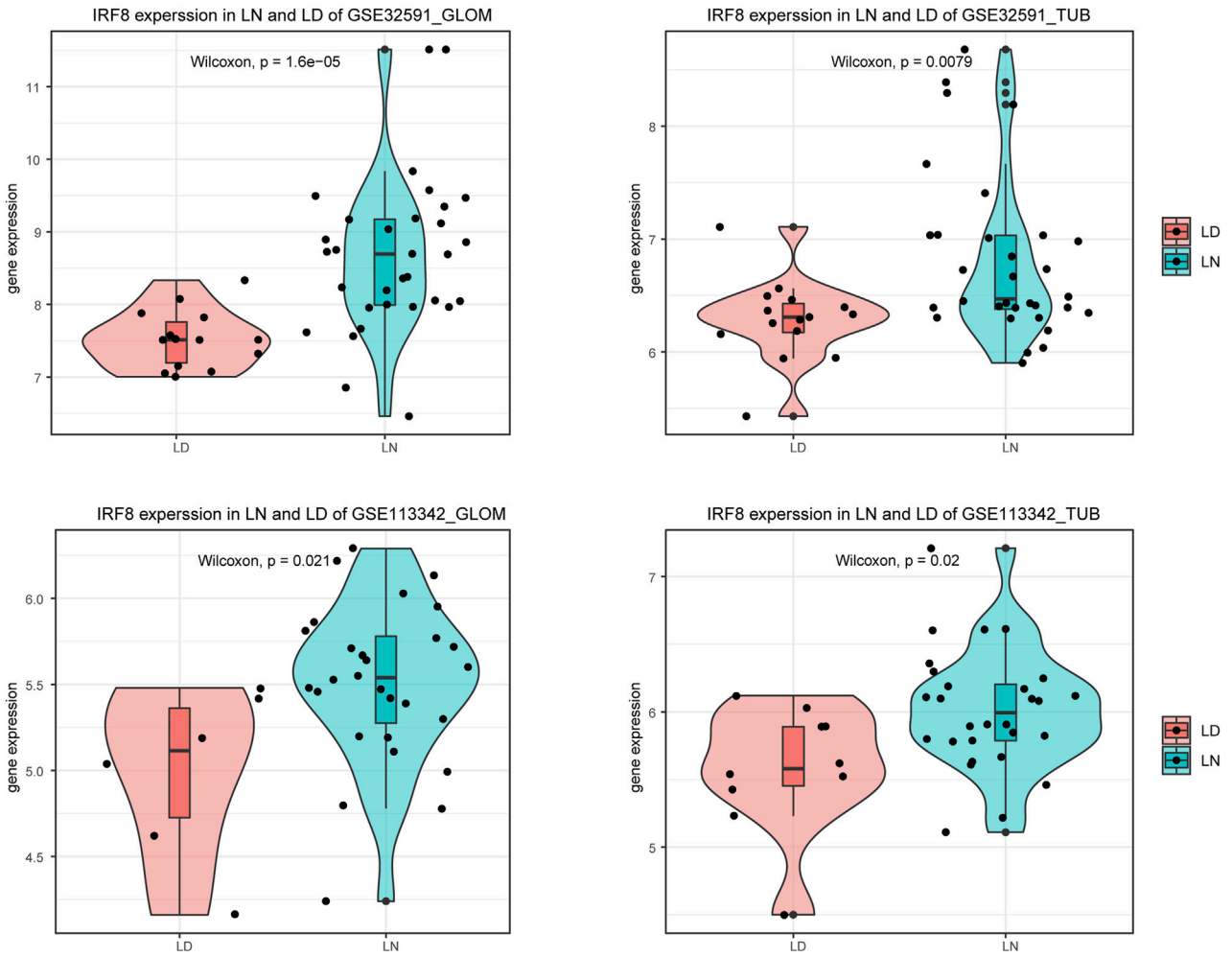

FIGURE 2
Expression levels of *IRF8* across datasets.

analysis of the 20 identified co-DEGs. These genes were primarily associated with BP, such as the regulation of complement activation, synapse pruning, regulation of the humoral immune response, and cell junction disassembly. Regarding MF, the co-DEGs were enriched in activities such as toll-like receptor binding, peptide binding, transcription coactivator binding, and amide binding. Furthermore, they were linked to CC, including blood microparticles, specific granules, secretory granule membranes, and collagen trimers (Figure 3A). The co-DEGs were also enriched in biological pathways, such as those involved in pertussis, *Staphylococcus aureus* infection, complement and coagulation cascades, Leishmaniasis, and Chagas disease (Figure 3B).

3.3 Construction of PPI network and module extraction

We used the STRING database to construct a PPI network (Figure 4A) consisting of 16 genes and 34 interaction pairs to explore the interactions between co-DEGs. Within this network, the IRF8 node exhibited the highest degree of connectivity. To explore the impact of IRF8 on LN, we calculated genes highly correlated with IRF8 expression levels and identified 1080 IRF8-related genes in GSE32591-GLOM, 1,373 in GSE32591-TUB, 96 in GSE113342-GLOM, and 108 in GSE113342-TUB. An intersection of the four IRF8-related gene sets revealed 35 genes significantly correlated with IRF8 across at least three datasets (Figure 4B). We extracted a PPI network for these 35 IRF8-related genes using the STRING database (Figure 4C), which included 35 genes and 139 interactions, encompassing 11 co-DEGs (Figure 4D). C1QA and C1QB, validated by the literature to be related to LN (Wu et al., 2020), were among these. This indicates a close correlation between IRF8-related genes and LN. Subsequent analysis of the biological functions affected by IRF8-related genes revealed that these 35 genes were mainly enriched in BP, such as regulation of immune effector processes, adaptive immune responses based on somatic recombination of immune receptors built from immunoglobulin superfamily domains, lymphocyte-mediated immunity, and neutrophil degranulation (Figure 4E). They were also enriched in CC-like specific granules, MHC protein complexes, specific granule membranes, and secretory granule membranes (Figure 4F) and MF,

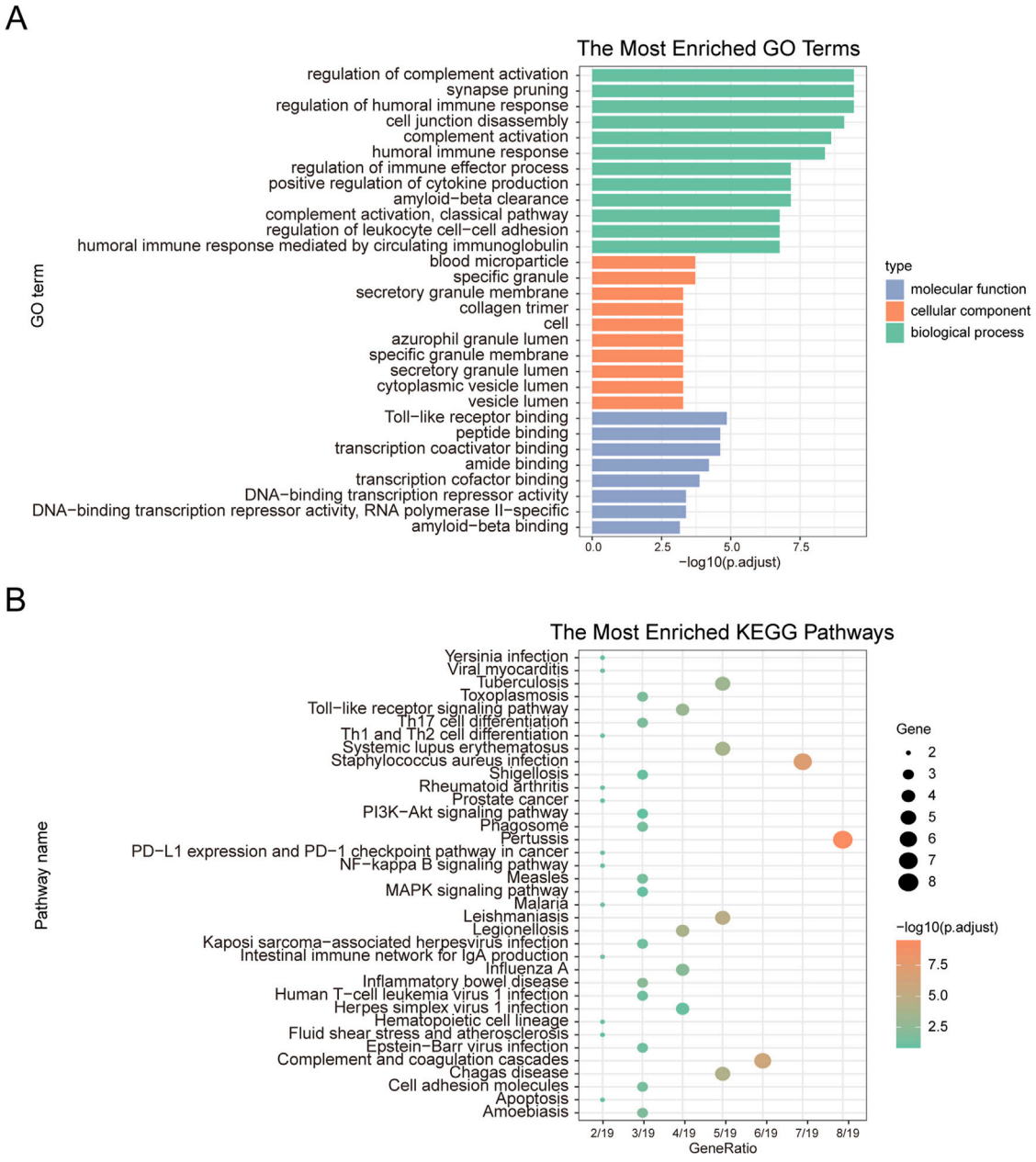

FIGURE 3
GO functional enrichment analysis and KEGG pathway enrichment analysis. **(A)** GO functional enrichment analysis. The x-axis represents -log(p.adjust), and the y-axis represents GO terms. **(B)** KEGG pathway enrichment analysis. The x-axis represents generation, and the y-axis represents pathway names. The size of the nodes indicates the number of genes enriched in the pathway, while the node color represents -log10(*p*-value).

including peptide, amide, peptide antigen, and integrin binding (Figure 4G). Furthermore, they affected biological pathways, such as *Staphylococcus aureus* infection, pertussis, complement and coagulation cascades, and cell adhesion molecules (Figure 4H).

3.4 Characteristic gene screening and diagnostic value assessment

By intersecting IRF8-related genes with the co-DEGs, we identified 11 key genes. To assess their diagnostic value for LN, we used the RandomForest feature selection method within WEKA on the GSE32591-GLOM dataset and constructed a diagnostic classifier using the RandomForest package in R, resulting in an importance score for the 11 feature genes (Figure 5A). We validated the diagnostic classifier using three datasets: GSE32591-TUB, GSE113342-GLOM, and GSE113342-TUB and plotted ROC curves. The results illustrated that the AUC for GSE32591-TUB was 0.738 (Figure 5B), for GSE113342-GLOM was 0.929 (Figure 5C), and for GSE113342-TUB was 0.914 (Figure 5D), indicating that our diagnostic classifier can effectively differentiate disease samples from normal samples.

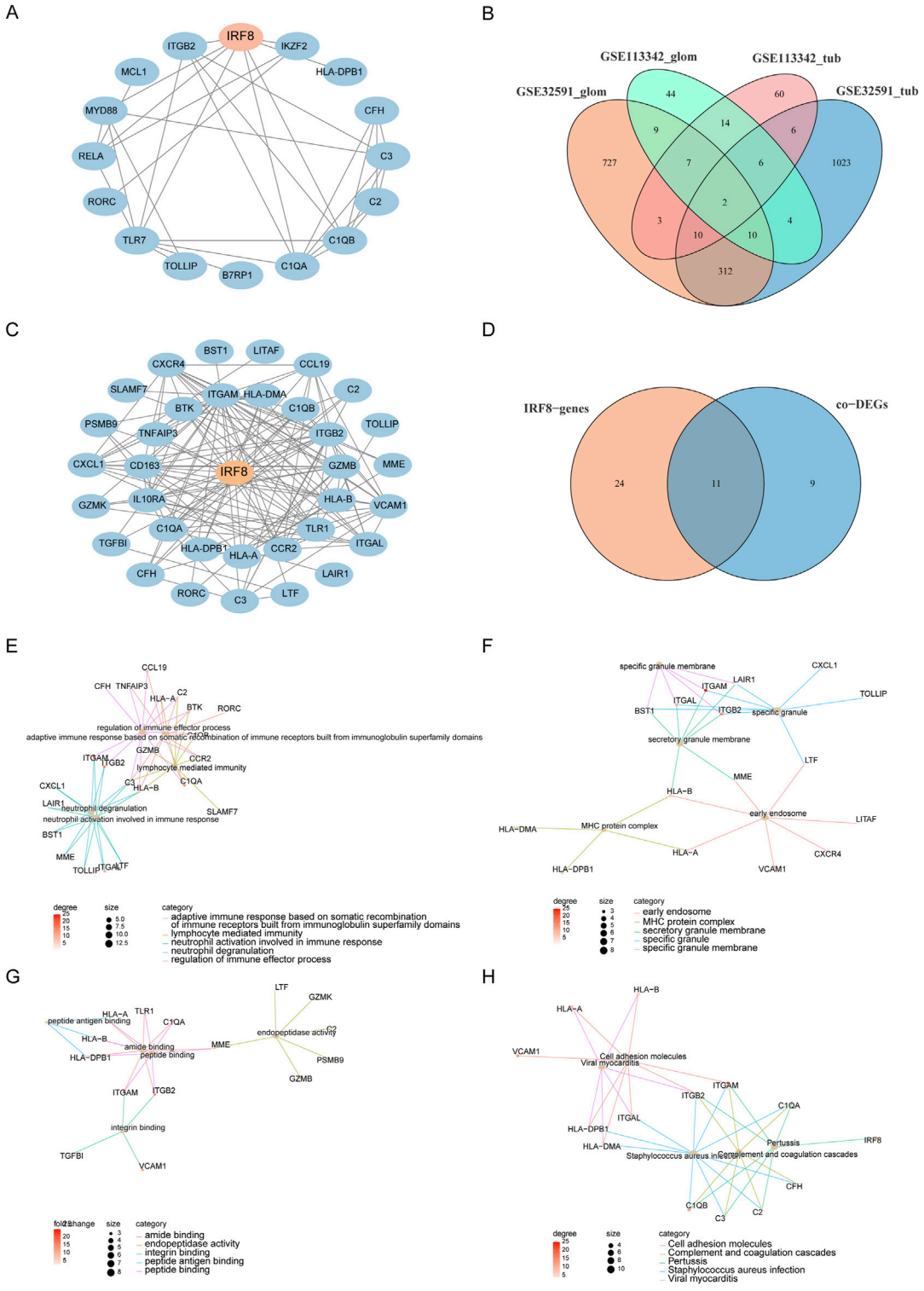

FIGURE 4
PPI and Functional Analysis. **(A)** PPI network of co-DEGs. **(B)** IRF8-related genes obtained from four datasets. **(C)** PPI network of IRF8-related genes. **(D)** Intersection of IRF8-related genes and co-DEGs. **(E–H)** Functional annotation and pathway enrichment analysis of genes in functional modules using DAVID, with node color representing the degree of IRF8-related gene nodes.

Furthermore, we analyzed whether IRF8 could distinguish diseased samples from normal samples. The results indicated that the AUC for IRF8 in GSE32591-GLOM was 0.877 (Figure 5E), in GSE32591-TUB was 0.744 (Figure 5F), in GSE113342-GLOM was 0.807 (Figure 5G), and in GSE113342-TUB was 0.752 (Figure 5H), respectively, demonstrating that the expression level of IRF8 significantly affected LN diagnosis.

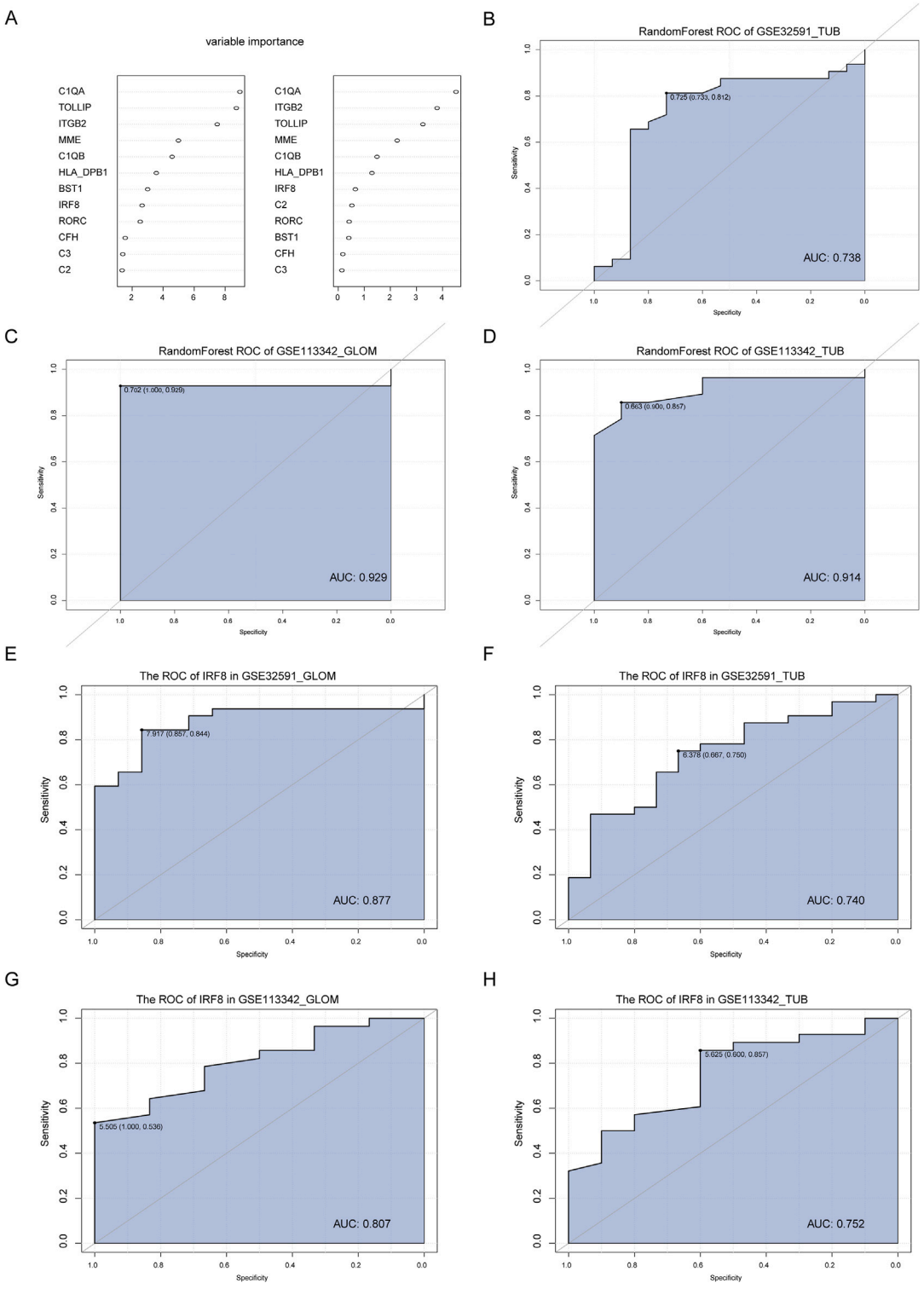

FIGURE 5
Construction and Validation of the Diagnostic Classifier. **(A)** Feature selection and diagnostic classifier model construction using the random forest algorithm on the GSE32591-GLOM dataset, ranking the importance scores of 11 key feature genes. **(B–D)** Testing the diagnostic classifier model on three additional datasets: GSE32591_TUB, GSE113342_GLOM, and GSE113342_TUB, with ROC curves plotted. **(E–H)** ROC curves of the IRF8 gene in GSE32591_GLOM, GSE32591_TUB, GSE113342_GLOM, and GSE113342_TUB datasets.

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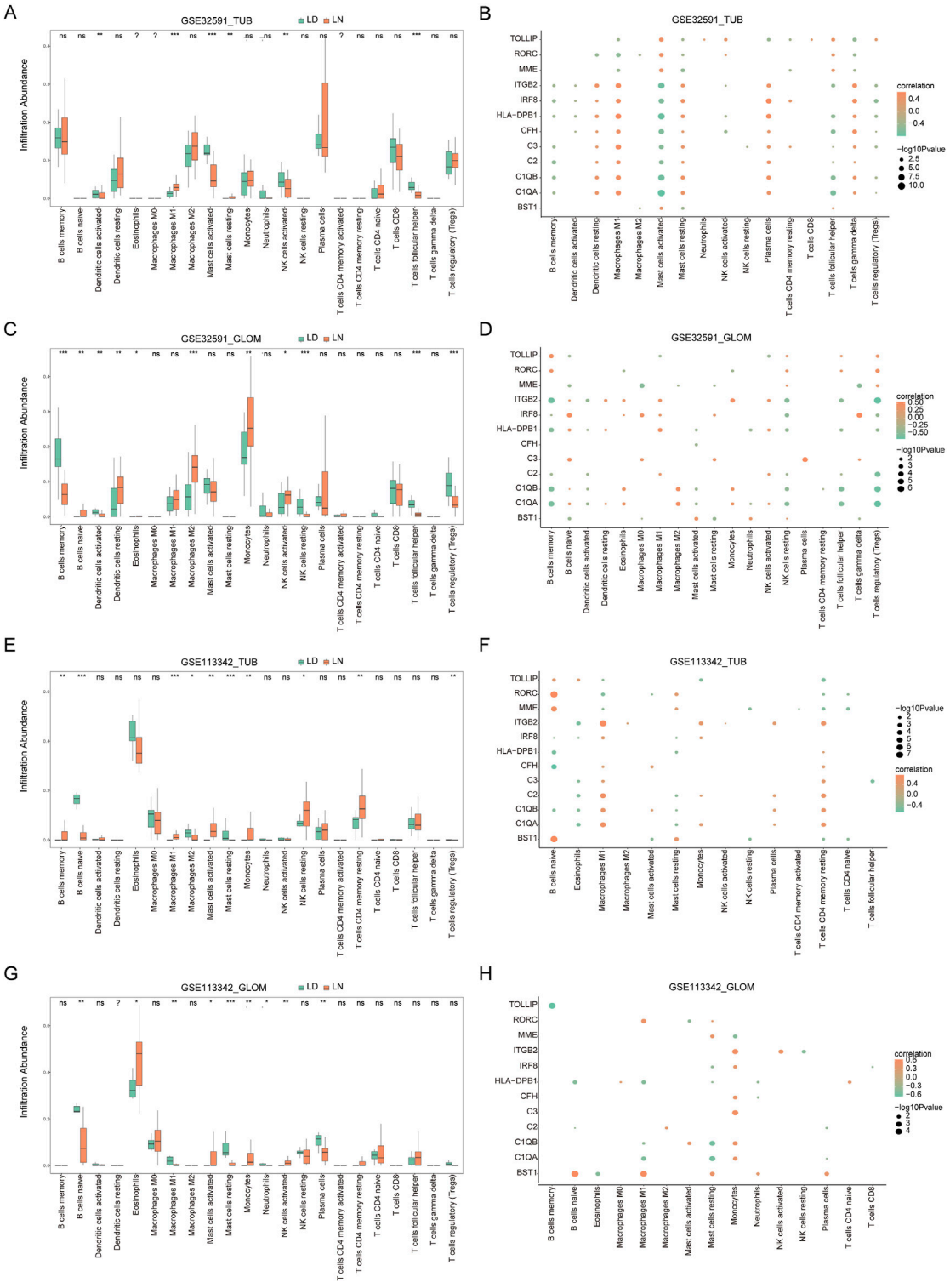

FIGURE 6
Immune Infiltration Analysis and Correlation between Key Genes and Immune Cells. **(A)** Immune infiltration in the GSE32591_TUB group, with the x-axis representing immune cells and the y-axis representing immune cell abundance. Asterisks indicate significance levels: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Similar plots are shown for GSE32591_GLOM **(C)**, GSE113342_TUB **(E)**, and GSE113342_GLOM **(G)**. **(B)** Correlation between 11 key feature genes and immune cells in the GSE32591_TUB group. The x-axis represents immune cells, and the y-axis represents feature genes, with orange indicating positive correlation and green indicating negative correlation. Node size represents the level of significance. Similar plots are shown for GSE32591_GLOM **(D)**, GSE113342_TUB **(F)**, and GSE113342_GLOM **(H)**.

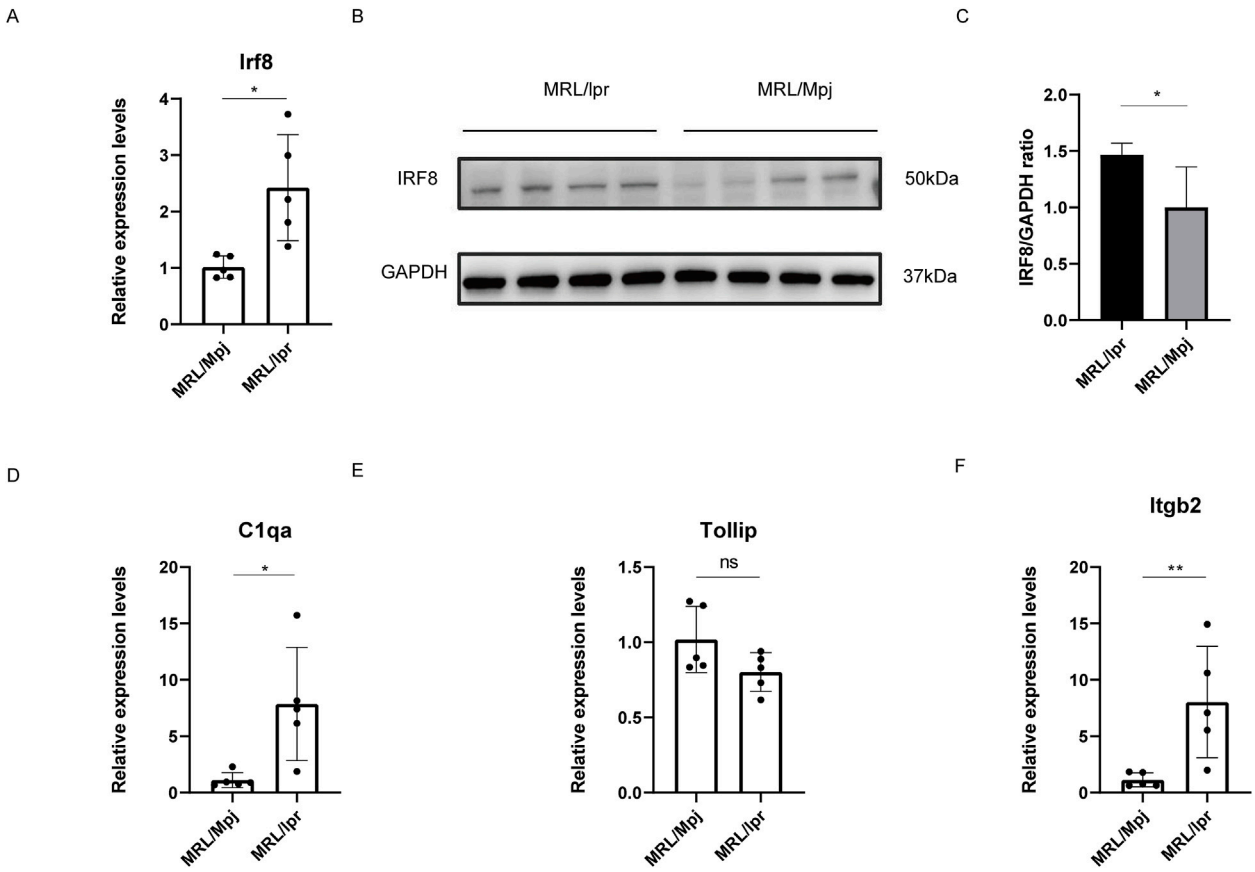

FIGURE 7
Validation of IRF8 Expression and Its Related Genes. **(A)** qRT-PCR validation for Irf8 (n = 5). **(B)** Western blot validation for IRF8 expression (n = 4). **(C)** The quantification of the Western blot bands of IRF8. **(D)** qRT-PCR validation for C1qa (n = 5). **(E)** qRT-PCR validation for Tollip (n = 5). **(F)** qRT-PCR validation for Itgb2 (n = 5). Significance levels are denoted as follows: *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, with 'ns' indicating no significant difference.

3.5 CIBERSORT immune infiltration analysis

The CIBERSORT algorithm was utilized to evaluate immune cell infiltration differences across two distinct RNA modification patterns. The analysis revealed substantial variations in immune cell populations between LN and LD groups. Specifically, in GSE32591-TUB, there were significant differences in the proportions of activated dendritic cells, M1 macrophages, activated and resting mast cells, activated NK cells, and follicular helper T cells ($p < 0.05$, Figure 6A). Moreover, a significant correlation was observed between key genes and the number of M1 macrophages and activated and resting mast cells (Figure 6B). In GSE32591-GLOM, memory B cells, naïve B cells, activated and resting dendritic cells, and eosinophils were significantly different ($p < 0.05$, Figure 6C), with memory and naïve B cells exhibiting significant correlations with key gene expression levels (Figure 6D). For GSE113342-TUB, significant differences were observed in the content of M1 and M2 macrophages, activated and resting mast cells, and monocytes ($p < 0.05$, Figure 6E); M1 macrophages and resting mast cells also demonstrated significant correlations with key gene expression levels (Figure 6F). Last, in GSE113342-GLOM, notable differences were detected in the levels of activated mast cells, resting mast cells, monocytes, neutrophils, and activated NK cells ($p < 0.05$, Figure 6G), with significant correlations between resting mast cells, monocytes, and multiple key genes.

3.6 Validation of IRF8 and levels of its related genes

Recent study have demonstrated that 18-week-old MRL/lpr mice displayed glomerular swelling and significant kidney inflammatory cell infiltration (Chen et al., 2023). Consequently, we assessed IRF8 expression levels in the kidneys of these mice using qRT-PCR and Western blot analysis (Figures 7A–C). The results showed that both gene and protein expression levels of IRF8 were significantly elevated in the kidneys of 18-week-old MRL/lpr mice compared to control mice. Additionally, we used qRT-PCR to evaluate the expression of C1qa, Tollip, and Itgb2 (Figures 7D–F). Our findings revealed that C1qa and Itgb2 expression levels were significantly upregulated, while Tollip showed no significant difference in the kidneys of 18-week-old MRL/lpr mice compared to control mice.

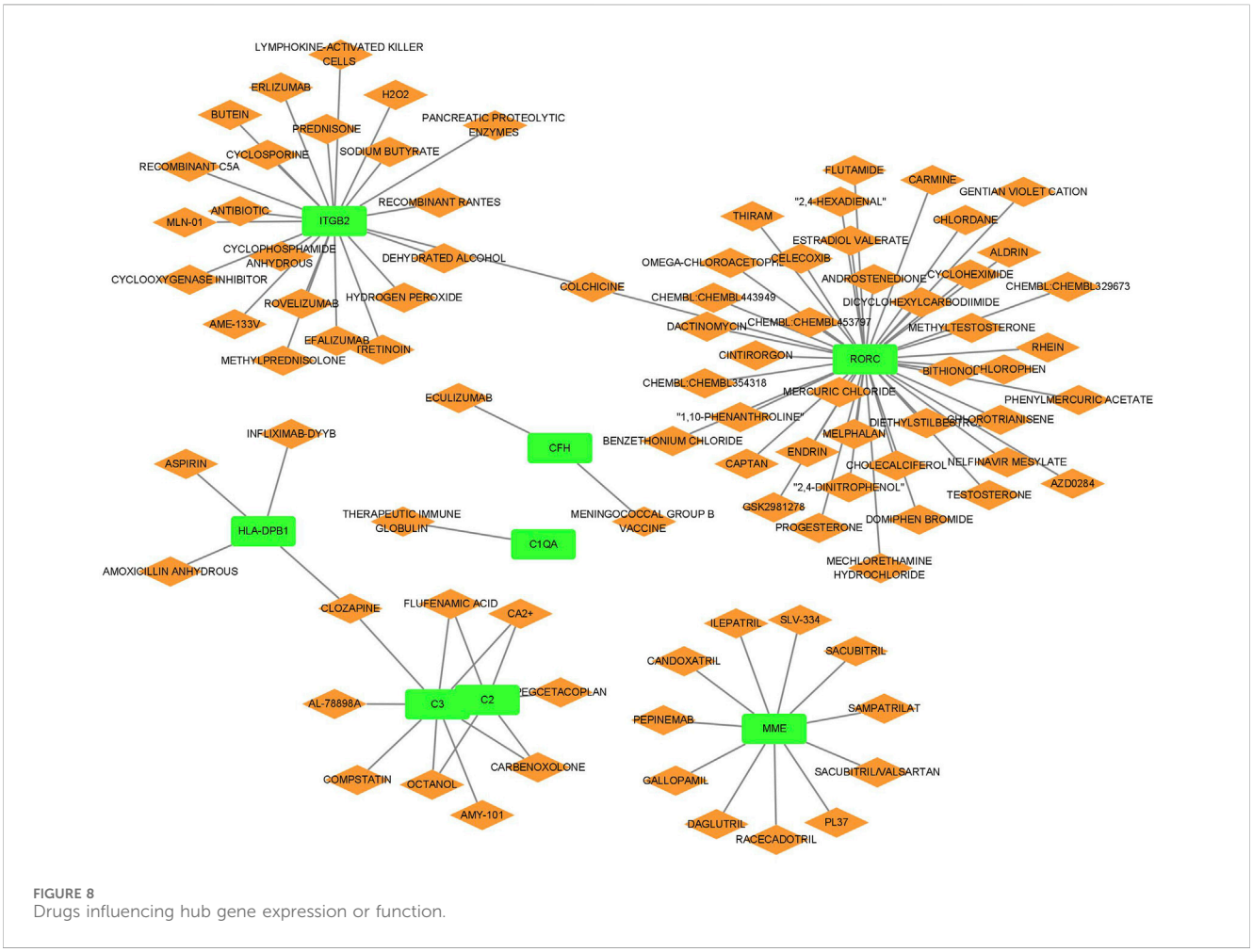

FIGURE 8
Drugs influencing hub gene expression or function.

3.7 Analysis of drug regulatory network in LN

The relationship between the biomarkers and drugs is presented in Figure 8. We identified several drugs targeting multiple genes integral to the disease pathway. Calcium ions (Ca2+), carbenoxolone, flufenamic acid, and octanol interacted with genes C2 and C3, suggesting a mechanism by which these drugs may influence the complement system, essential for immune responses. Similarly, clozapine targeted HLA-DPB1 and C3, potentially indicating its role in modulating immune functions and inflammatory responses. Furthermore, colchicine interacted with ITGB2 and RORC, underscoring its potential to regulate cellular adhesion processes and immune cell differentiation pathways. These insights suggest that these drugs may play a significant role in comprehensive strategies to treat conditions involving these critical pathways.

4 Discussion

LN affects ≤40% of all adults and ≤80% of all children with SLE and causes irreversible kidney damage. However, its pathogenesis is unclear, and no specific or sensitive biomarkers exist for its diagnosis or treatment. In clinical trials, only 30%–50% of patients enter remission, and 10%–20% develop ESRD within 10 years of diagnosis (Maria and Davidson, 2020). Therefore, it is vital to understand the pathology and molecular mechanisms underlying LN for its effective diagnosis and treatment. Microarray and bioinformatics analyses can clarify the molecular mechanisms underlying disease occurrence and development. In our study, by intersecting the DEGs across the datasets, we identified 20 co-DEGs. Further intersecting IRF8-related genes with these co-DEGs led us to identify 11 key genes. IRF8 was significantly upregulated in both GLOM and TUB groups. As a key transcription factor, IRF8 is vital for innate and adaptive immunity and contributes to cytokine production, particularly in the type I interferon pathway. These cytokines may lead to aberrant immune cell activation, resulting in the chronic inflammation commonly observed in SLE (Salloum and Niewold, 2011). Moreover, variations in the IRF8 gene increase susceptibility to SLE by regulating immune responses to environmental triggers (Cunninghame Graham et al., 2011; Lin et al., 2015; Sheng et al., 2015; Cai et al., 2017). This gene association highlights the potential of IRF8 as a biomarker for assessing SLE risk and disease progression. However, despite substantial research into the role of IRF8 in SLE, few studies have explored its involvement in LN, and its function in LN remains unclear. The complement system also plays a crucial role in SLE pathogenesis, particularly components such as C1QA, C1QB, C2, and C3, which are

essential for clearing apoptotic debris and immune complexes, thereby reducing autoimmunity and systemic inflammation (Mitchell et al., 2002; Sun-Tan et al., 2010; Carlucci et al., 2016). Genetic polymorphisms in C1QA and C1QB have been associated with increased SLE susceptibility, affecting serum C1q levels and disease severity. The rs631090 SNP in the C1QB gene is linked to SLE, leading to lower C1q levels, which may result in inefficient clearance of immune complexes and apoptotic cells (Martens et al., 2009). C2 and C3 are key complement system components and are integral to the classical and alternative pathways. C2 deficiency impairs immune complex clearance and increases SLE risk (Lundtoft et al., 2022). Serum C3 levels are significantly heritable and identifies specific genetic variants within the C3 gene associated with both serum levels and SLE susceptibility (Rhodes et al., 2009). In our analysis, the enrichment of the complement and coagulation cascade pathways further supports the critical role of the complement system in SLE. Complement factor H (CFH) primarily regulates the alternative complement pathway and prevents uncontrolled complement activation and tissue damage. Studies have demonstrated that CFH deficiency models exhibit severe disease progression, with increased proteinuria, elevated BUN levels, and significant kidney damage due to uncontrolled complement activity and immune complex deposition. Despite their crucial role, cross-population genetic studies suggest that individual genetic variations may not significantly affect disease susceptibility or progression (Bao et al., 2011; Li Q.-Y. et al., 2023; Ma et al., 2023). Retinoic acid-related orphan receptor C (RORC) primarily functions in Th17 cell differentiation and may contribute to SLE pathogenesis by regulating interleukin (IL)-17 production. RORC expression is lower in patients with SLE compared to healthy individuals, suggesting an imbalance in immune regulation, particularly in the interaction between IL-23 and STAT3, which may influence clinical symptoms and treatment outcomes in SLE (El-Karaksy et al., 2016; Kluger et al., 2017). The ITGB2 gene encodes the β2 integrin subunit, a key component of the β2 integrin family that promotes cell adhesion and immune responses (Li H. et al., 2023). Membrane metalloendopeptidase, also known as neprilysin, is involved in various physiological and pathological processes, including cancer and autoimmune diseases (Ding et al., 2023). HLA-DPB1 alleles are associated with SLE and specific autoantibodies. Some studies have found that certain HLA-DPB1 alleles are related to anticardiolipin and anti-β2 glycoprotein I antibodies, suggesting their involvement in autoimmune responses. Additionally, these alleles are associated with specific clinical features of SLE, such as livedo reticularis and Raynaud's phenomenon, further contributing to the clinical diversity of the disease (Korioth et al., 1992; Sebastiani et al., 2003). To date, no studies have investigated the relationship between BST1 and TOLLIP genes and SLE.

By integrating IRF8-related genes with the co-DEGs, we identified 11 key genes and assessed their potential as diagnostic markers for LN using the random forest algorithm in WEKA, based on the GSE32591-GLOM dataset. We tested the diagnostic classifier on three independent datasets with AUC values of 0.738, 0.929, and 0.914 for GSE32591-TUB, GSE113342-GLOM, and GSE113342-TUB, respectively. These results indicate that this method can effectively distinguish the disease. Additionally, the diagnostic significance of IRF8 was confirmed, with AUC values ranging from 0.744 to 0.877 across the different datasets.

We further explored the effect of co-DEGs on immune infiltration in LN. The analysis of immune cell variations between the LN and control groups revealed significant differences. B cells are integral to the pathogenesis of SLE, primarily through autoantibody production, antigen presentation, and immune response modulation. Abnormalities in B-cell tolerance, signaling, and cytokine production contribute to atypical B-cell activation and differentiation, advancing disease progression (Yap and Chan, 2019). Macrophages and dendritic cells are essential for SLE, specifically in the LN. Macrophages originate from monocytes and are crucial for phagocytosis, tissue remodeling, and cytokine production. In LN, renal macrophages, particularly the resident F4/80^{hi} population, proliferate and assume an inflammatory phenotype that causes tissue damage and fibrosis. Efficient antigen-presenting dendritic cells infiltrate the kidneys and form tertiary lymphoid structures, exacerbating the local inflammation. The disrupted functions of these cell types in SLE heighten immune responses and impede resolution; therefore, they are identified as primary targets for therapeutic strategies to preserve renal function and mitigate disease progression (Maria and Davidson, 2017).

This study, primarily focused on validating the differential expression of the IRF8 gene in lupus mouse models, encounters several limitations. It does not include validation in human tissues nor does it explore the specific roles of the IRF8 gene in LN. Additionally, the tissue sample size is insufficient, requiring enlargement to more robustly confirm and generalize the findings. Lastly, the conclusions of the study are based exclusively on a single type of omics analysis, which might overlook essential biological interactions and pathways that could be uncovered through a comprehensive multi-omics approach.

5 Conclusion

In conclusion, this study has successfully identified IRF8 and IRF8-related genes that possess significant diagnostic value for LN. This research provides novel insights into the diagnosis and treatment of LN and lays a solid foundation for future empirical investigations.

Data availability statement

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

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee of Fudan University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ZY: Writing–original draft, Writing–review and editing. CZ: Writing–review and editing. YW: Writing–review and editing.

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Conflict of interest

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

Publisher's note

Supplementary material

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Hepatobiliary organoid research: the progress and applications

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Organoid culture has emerged as a forefront technology in the life sciences field. As "*in vitro* micro-organs", organoids can faithfully recapitulate the organogenesis process, and conserve the key structure, physiological function and pathological state of the original tissue or organ. Consequently, it is widely used in basic and clinical studies, becoming important preclinical models for studying diseases and developing therapies. Here, we introduced the definition and advantages of organoids and described the development and advances in hepatobiliary organoids research. We focus on applying hepatobiliary organoids in benign and malignant diseases of the liver and biliary tract, drug research, and regenerative medicine to provide valuable reference information for the application of hepatobiliary organoids. Despite advances in research and treatment, hepatobiliary diseases including carcinoma, viral hepatitis, fatty liver and bile duct defects have still been conundrums of the hepatobiliary field. It is necessary and crucial to study disease mechanisms, establish efficient and accurate research models and find effective treatment strategies. The organoid culture technology shed new light on solving these issues. However, the technology is not yet mature, and many hurdles still exist that need to be overcome. The combination with new technologies such as CRISPR-HOT, organ-on-a-chip may inject new vitality into future development.

KEYWORDS

organoid, stem cells, hepatobiliary disease, disease modeling, personalized medicine (PM)

1 Introduction

1.1 Definition of organoids

Organoids are described as intricate 3-dimensional (3D) structures originating from human (pluripotent) stem cells, progenitor, and/or differentiated cells. They have the ability to self-assemble and differentiate into functional clusters of multiple cells, accurately reproducing the function, organization, and genetic characteristics of the original organs *in vivo* (Dutta et al., 2017; Marsee et al., 2021). Traditional 2-dimensional (2D) cell culture technique is used the most and has the advantages of convenience and simplicity. However, 2D attachment leads to cells losing their morphology and influences the organization of the structures inside the cell, proliferation, growth and differentiation, secretion, signal transduction and drug response (Baker and Chen, 2012; Scalise et al., 2021), with heterogeneity gradually obliterated, genomics and metabolomics significantly dissimilated during long-term subculture (Bresnahan et al., 2020). The organoid technology, as a 3D culture system, is created through suspension culture to

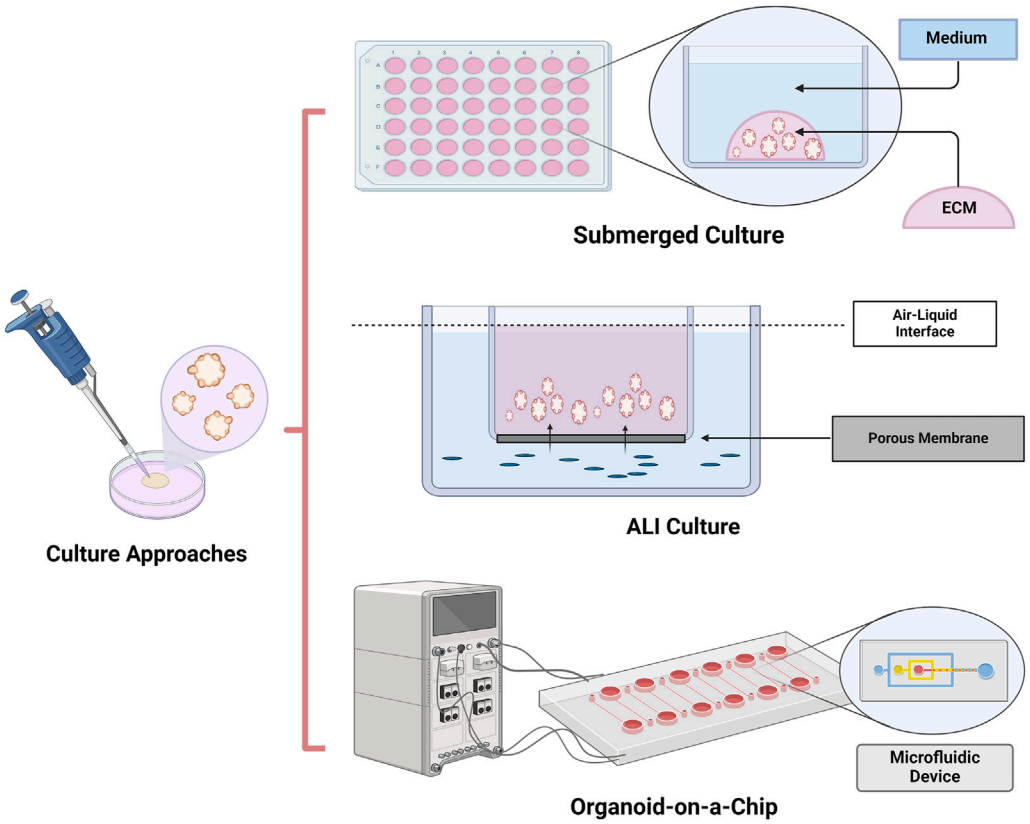

FIGURE 1
Culture approaches of organoids. Organoids can be achieved by submerged culture, air–liquid interface (ALI) culture and Organoids-on-a-chip culture. Submerged culture is the most widely used organoids culture method. Because of direct exposure to oxygen, ALI cultures provide higher oxygen supplement than submerged culture. Organ-on-a-chip is a microfluidic cell culture device which could accurately control the abiochemical and biophysical environment for cell growth.

prevent direct physical contact with the plastic dish, in contrast with the 2D culture method. The establishment of the 3D environment mainly relies on biological or synthetic scaffolds similar to the extracellular matrix. Furthermore, scaffold-free methods (Dituri et al., 2021), "air-liquid-interface" methods (Scalise et al., 2021; Neal et al., 2018; Lamers et al., 2021; Wakamatsu et al., 2022) and "Organ-on-a-chip" (Xue et al., 2021; Huang et al., 2021; Xie et al., 2022) can also be utilized to attain the 3D structure of the organoids (Figure 1). The intricate surrounding milieu regulates the structure, development and function of cells in the organism, encompassing interactions between cells and cell-extracellular matrix (ECM). Because Matrigel plays an excellent supporting role, the 3D culture conditions can recapitulate the microenvironment in which primary cells are located accurately (Tuveson and Clevers, 2019). In this scenario, 3D-grown organoids exhibit strong resemblance to the parents, and also retain the genetic stability and chromatin heterogeneity of the parents. Additionally, organoids can proliferate quickly within 1–2 weeks and can be stably sub-cultured and cryopreserved similar to normal cell lines (Drost and Clevers, 2018). In addition, cells are able to aggregate into spherical shapes under 3D culture conditions, which contributes to establishing the intercellular signaling pathways (Fan et al., 2019). Organoid models have characteristics similar to living organs: 1) they contain various organ-specific cell types; 2) exhibiting some specific functions related to organs; 3) forming a spatial structure similar to organs. Organoid are considered as an important model in exploring the occurrence, progression and evolution of diseases due the ability of faithfully replicating and simulating the distinctive biological traits of organs and parent cells. Moreover, tumor organoids can be established through preoperative biopsy or postoperative resection specimen, serving a crucial function in predicting personalized drug sensitivity and screening adjuvant therapy medications. Therefore, organoid models offer superior alternatives for drug screening and personalized drug treatment (Broutier et al., 2017; Wang et al., 2021; Yuan et al., 2022). The recognition of organoids' potential to broaden fundamental research by supplementing existing model systems is becoming more widespread (Bahmad et al., 2021).

Stem cells, being primitive and undifferentiated, possess the capability to differentiate into distinct and specialized cell categories. Organoids can be derived from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and adult stem cells (ASCs), due to the self-renewal and multi-directional differentiation potential of stem cells (Figure 2) (Lancaster and Knoblich, 2014; Takebe and Wells, 2019). The development of organoids from stem cells is comparable to how the organ obtains its unique organizations, primarily involves the self-organization of the cell population (Rossi et al., 2018). It needs

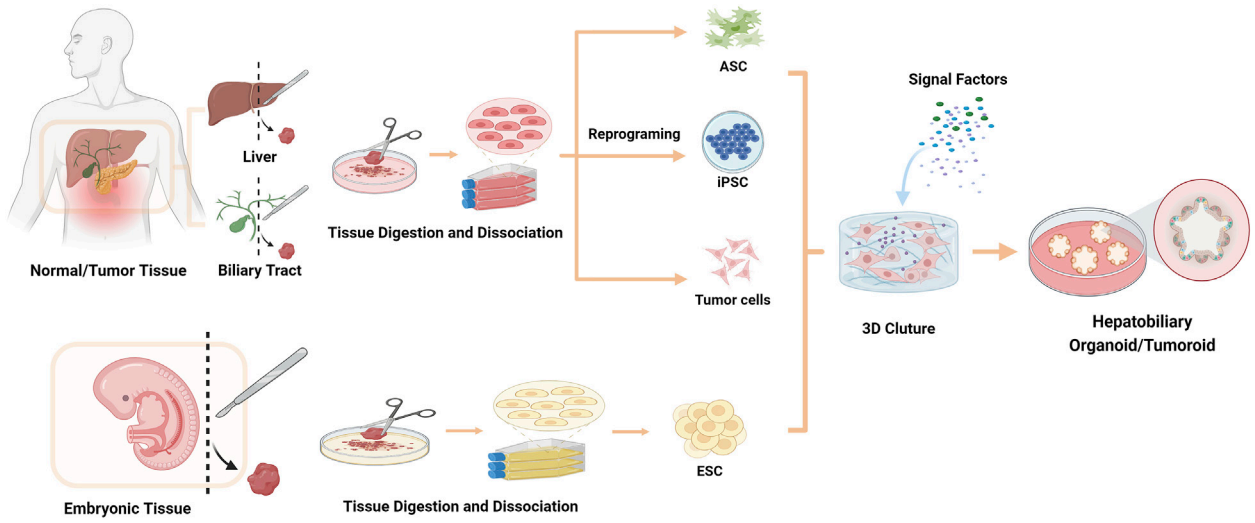

FIGURE 2
Strategies for organoids/tumoroids establishment *in vitro*. The cell sources for establishing organoids include ESCs, ASCs, iPSCs and tumor cells.

to mimic an *in vivo* microenvironment and active various signaling pathways during cell development and differentiation to induce self-organization. Organoids derived from pluripotent stem cells (PSCs, including ESCs or iPSCs) are established through directed differentiation of PSCs. To initiate cell-directed differentiation and maturation, it is necessary to form particular germ layers (endoderm, mesoderm or ectoderm) and then co-culture them with specific growth and signaling factors as well as cytokines. Culturing of ASCs-derived organoid require to isolate the tissue-specific stem cells from the target organ, and then embed them into an ECM containing defined, tissue-specific combinations of growth factors to support propagation (Huch and Koo, 2015; Kim et al., 2020). While initial studies suggested organoids were solely derived from stem cells (Lancaster and Knoblich, 2014), it is now evident that organoids can also originate from differentiated cells like cholangiocytes (Aloia et al., 2019; Sampaziotis et al., 2017).

Organoids are categorized into distinct groups based on defining characteristics, according to the consensus on the definition and nomenclature of hepatic, pancreatic and biliary organoids. These encompass epithelial, multi-tissue, and multi-organ ones. Epithelial organoids represent the most widely studied organoid type. These structures originate from one germ layer (endoderm, mesoderm, or ectoderm) and can self-renew under suitable culture conditions. Multi-tissue organoids are formed by co-culturing cells from two or more germ layers or by co-differentiating PSCs. Multi-organ organoids represent the most intricate and least documented category of organoids, characterized by inter-organ developmental self-organization patterns. These systems offer significant potential for researching organogenesis, a process regulated by various boundary tissue interactions (Marsee et al., 2021).

1.2 The current status

After Clevers' team in the Netherlands published their findings in 2009, reporting that leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) positive ASCs in the mouse intestine were capable of forming the crypt-villus structure *in vivo*, organoid models of normal organs and tumor tissues can be observed in studies of multiple fields, including the stomach, colon, pancreas, kidney, prostate, brain, and retina (Barker et al., 2010; Spence et al., 2011; Sato et al., 2011; Gao et al., 2014; Eiraku and Sasai, 2011; Lancaster et al., 2013; Low et al., 2019; Boj et al., 2015).

In 2013, the 3D culture method was used by Takebe's lab in the construction of 3D vascular and functional iPSC-derived liver buds (iPSC-LBs) *in vitro*. The analysis of immunostaining and gene expression demonstrated the resemblance between liver buds induced *in vitro* and those *in vivo*. Moreover, the internal functional blood vessels could promote the maturation of iPSC-LBs into liver tissue (Takebe et al., 2013). Takebe's study addressed the technical challenges of organoid boundary system formation, opening up the possibility of studying complex interactions during early organ development.

In the same year, Clevers et al. extracted $Lgr5^+$ progenitor-like oval cells from the portal triad area in injured mouse livers that CCl_4 induced. Then, $Lgr5^+$ cells were cultured and induced to differentiate in a Matrigel matrix with Wnt3a, R-pondin-1, EGF, HGF, FGF10 and Noggin to establish mature mouse hepatobiliary organoids (Huch et al., 2013). Certain liver progenitor cells could develop into early hepatocytes and biliary epithelial cells, according to the analysis of the resulting organoids. Which demonstrated that the mouse liver organoids were bipotential. Under the culture conditions that added Notch, TGF-β pathway inhibitors, FGF, BMP7, EGF, and dexamethasone without R-spondin-1 and HGF, these bipotential organoids tended to differentiate into hepatocytes. After implantation into immunodeficient mice, mouse liver organoids differentiated into liver tissues and showed mature hepatocyte markers and function, including low-density lipoprotein uptake, albumin and bile acid secretion, glycogen accumulation, and induction of the cytochrome P450 system (Huch et al., 2013; Schulze et al., 2019). Soon after in 2015, Clevers et al. successfully established human liver organoids originated from $EpCAM^+$ cells obtained from the human liver

in vitro, which were bipotential (Huch et al., 2015). In 2018, studies showed that more mature and longer-lasting hepatocyte organoids could be constructed by inducing purified AXIN2$^+$ mouse hepatocytes (Peng et al., 2018; Hu et al., 2018). Furthermore, there have been reports of hepatocyte organoids originated from human embryonic liver tissue of aborted fetuses (Hu et al., 2018). Wang et al. established human ESCs derived expandable hepatic organoids (hEHOs) from using a new type of media (serum-free, feeder-free). The hEHOs were capable of maintaining the phenotypic traits of bipotential hepatic stem cells stably and had the ability to differentiate into functional hepatocytes or cholangiocytes (Wang et al., 2019). Wu et al. successfully established the first functional hepatobiliary organoids (HBOs) using human induced pluripotent stem cells (hiPSCs) (Wu et al., 2019). The authors produced hepatobiliary organoids by inducing hiPSCs to form endoderm and mesoderm tissues simultaneously and activating the NOTCH2 and TGF-β signaling pathways to generate separate hepatocyte and cholangiocyte populations. Next, the hepatobiliary organoids were matured using a proprietary cholesterol$^+$ MIX supplemented standard base medium. Soon after, Wu et al. updated their previous protocol (Wu et al., 2019), which shortened the time to achieve maturation *in vitro*, and developed a medium that could maintain HBOs for more than 1.5 months (Wu et al., 2021). Takebe's team successfully constructed the continuous and dynamic hepato-biliary-pancreatic organoid (HBPO). Furthermore, a functional connection between the internal pancreas, especially the exocrine lineage, and the bile ducts within HBPO (Koike et al., 2019). Functioning human liver organoids were generated from pluripotent stem cells derived from peripheral blood CD34$^+$ cells by Kasem et al. (Kulkeaw et al., 2020). Since only the hepatic endoderm was able to form liver organoids without co-culture with the endothelium and septum mesenchyme, endothelial cells or hepatic progenitor cells (Takebe et al., 2013; Pettinato et al., 2019; Ng et al., 2018), the method of Kasem et al. was simple and faster than a previous study (Mun et al., 2019). This study also showed that hiPSCs produced from hematopoietic progenitor cells could differentiate into hepatocytes and create liver organoids, indicating that a less invasive approach could be used to manufacture hiPSCs. Wendy et al. constructed multi-cellular human liver PSC-derived organoids, comprised predominantly hepatic epithelial cells, differentiated simultaneously with stellate-like and hepatic macrophage-like cell that had the potential for modeling of hepatic inflammatory diseases *in vivo* (Thompson and Takebe, 2020).

Research have shown that self-renewing epithelial organoids can be cultured from primary tissue of the human liver (Huch et al., 2015; Hu et al., 2018) and extrahepatic biliary tree (Sampaziotis et al., 2017; Lugli et al., 2016). Self-organizing 3D structures could also be cultured from primary and metastatic tumors and even tumor needle biopsies of the liver and extrahepatic bile ducts (Broutier et al., 2017; Nuciforo et al., 2018; Saito et al., 2019). Hepatocellular carcinoma (HCC)-derived organoids replicate the histological structure, mutation profile, and transcriptome of the original tumor. The same applied to the culture of intrahepatic cholangiocarcinoma organoids, which maintained their drug-resistance phenotype, enabling in-depth mechanistic and personalized drug interaction research.

2 Applications of hepatobiliary organoids

Organoid technology has significant advantages: 1) Human-derived: Human organoids represent human physiology, 2) Rapid: Organoids can be rapidly and easily established derived from ASC and PSC, 3) Robustness: Scale-up is usually possible for drug and genomic screening on a large scale, once established, 4) Genetic manipulation: majority of genetic engineering tools can be used on iPSC or directly on organoid systems, 5) Personalization: iPSCs and organoids can be obtained from individuals (Kim et al., 2020). The wide array of biomedical applications (Figure 3) is facilitated by these benefits of hepatobiliary organoids.

2.1 Hepatobiliary disease research

2.1.1 Hereditary disease

Organoids are able to be used to study and model organ-specific genetic diseases. Alpha-1 antitrypsin (AAT) deficiency (AATD), one of the inherited metabolic diseases, results from deficiency of the anti-protease component-α1-antitrypsin in the blood. Clevers et al. used biopsies from patients with α1-antitrypsin deficiency to generate liver organoids by organoid culture technology (Huch et al., 2015). They observed AAT protein aggregates in the resulting organoids, which resembled the findings in the original biopsy. Besides, supernatants from these organoids showed a lower ability to block elastase activity. In addition, a Spanish team' success confirming Clevers's findings (Gómez-Mariano et al., 2020).

Alagille syndrome (ALGS) is a rare multisystem disorder caused by mutations of the JAG-1 and NOTCH2 genes (ShenTu et al., 2021; Mitchell et al., 2018). The main hepatobiliary presentations are biliary atresia and chronic cholestasis caused by bile duct hypoplasia. The team of Clevers reported that they had established the first human ALGS liver organoid models (Huch et al., 2015). When R-spondin, Nicotinamide, TGFbi and FSK were removed, the ALGS liver organoids lost the potential to upregulate biliary markers. A similar conclusion was drawn from the study of Emma et al. (Andersson et al., 2018). Guan et al. introduced the mutation causing ALGS in JAG1 with Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) technology and cultured and induced iPSCs from healthy people to produce liver organoids whose pathological features were similar to ALGS (Guan et al., 2017).

Polycystic liver disease, also known as cystic fibrosis (CF), caused by mutations in a cell-surface chloride transporter called cystic fibrosis transmembrane regulator (CFTR) gene (Masyuk et al., 2022; Kothadia et al., 2024). Sampaziotis' team applied 3D culture technology to construct cystic fibrosis organoid models with cholangiocytes generated by inducing PSCs (Sampaziotis et al., 2015). Monique et al. established cholangiocyte organoids by extrahepatic cholangiocytes obtained from a compound CFTR gene mutation patient (Verstegen et al., 2020).

Wilson's disease, also known as hepatolenticular degeneration, is an autosomal recessive copper metabolism disorder, manifest as accumulation of copper ions in major organs such as the liver (Lucena-Valera et al., 2021; Arai et al., 2021). Nantasanti et al. segregated hepatocytes from

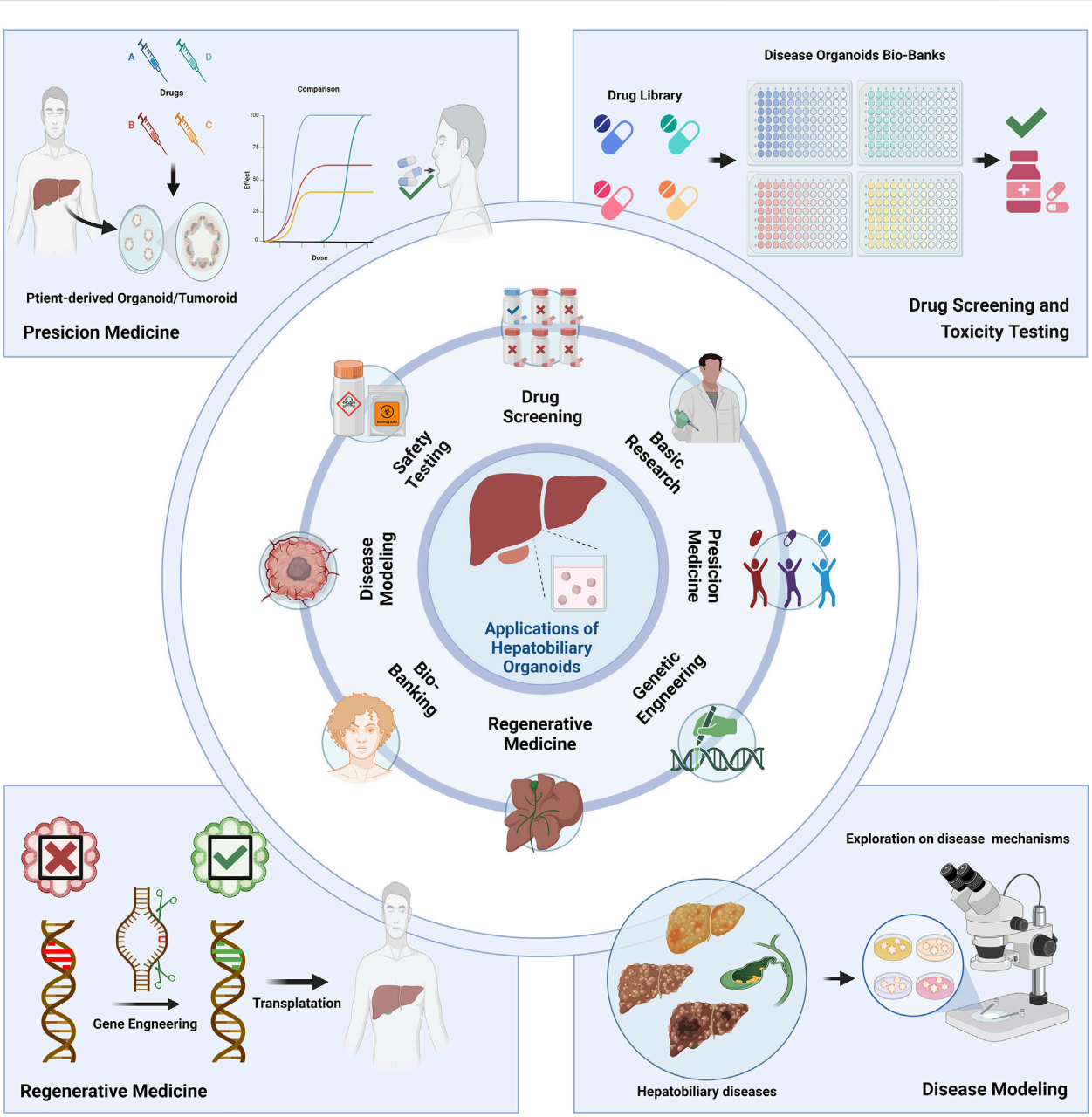

FIGURE 3
Applications of the hepatobiliary organoids. Organoids have wide application prospects on basic research, drug screening, safety testing, disease modeling, bio-banking, regenerative medicine, genetic engineering, precision medicine and many other fields.

copper metabolism MURR1 domain 1 (COMMD1) deficient dogs to culture organoids and observed the intracellular copper accumulation, which demonstrated that the *in vitro* model of Wilson's disease was generated successfully (Nantasanti et al., 2015). In 2020, a study isolated and cultured hepatic progenitors from COMMD1-deficient dogs to generate organoids (Kruitwagen et al., 2020). After gene correction, the organoid-derived hepatocyte-like cells are transplanted via the portal vein into the dog livers, and the cells engraft and survive up to 2 years. This study provided a new approach that applied organoids as tools to treat gene-defective inherited liver diseases.

Wolman disease (WD) is an autosomal recessive genetic disease caused by the inactivation of acid lipase in the lysosome (Aguisanda et al., 2017). A large amount of lipid accumulates in the hepatocytes contribute to steatohepatitis and fibrosis (Aguisanda et al., 2017; Pastores and Hughes, 2020). To explore new treatment methods, Ouchi et al. constructed three organoid models with severe fibrosis by culturing and inducing iPSCs of Wolman disease patients (Ouchi et al., 2019). They added FGF19, which could relieve symptoms of WD into the culture system of organoids and found that the production of reactive oxygen species, a marker of hepatocyte injury in nonalcoholic fatty liver, was significantly decreased (Attia et al., 2017).

2.1.2 Viral hepatitis

Viral hepatitis, especially hepatitis B and hepatitis C, is one of the major public health problems and economic burdens worldwide (Fotiadu et al., 2004; Shiha et al., 2020; Zhang et al., 2021). Two studies had used pluripotent stem cells from hepatitis B patients and liver specimens from hepatitis C patients to induce and culture liver organoids with hepatitis B and hepatitis C, respectively (Nie et al., 2018; Baktash et al., 2018). According to recent studies, liver organoids obtained from healthy individuals were co-cultured with either the recombinant virus or the serum of patients with HBV. As a result, the organoids became infected and the virus showed active proliferation (De Crignis et al., 2021). The 3D organoid models of viral hepatitis can demonstrate the connection and interaction between the hepatitis virus and host cells, offering essential preclinical models for mechanism research, drug discovery and treatment of viral hepatitis.

2.1.3 Fatty liver

Recently, organoids have been recognized as the favored 3D *in vitro* representation for studying non-alcoholic fatty liver disease (NAFLD) (Ramos et al., 2022). Ouchi' team, using PSC lines, developed a reproducible method to derive multi-cellular human liver organoids composed of hepatocyte-, stellate-, and Kupffer-like cells. Under treatment of free fatty acid, organoids recapitulated steatosis, inflammation and fibrosis phenotypes, which are key characteristics of steatohepatitis, successively. Gurevich et al. established a novel *in vitro* differentiation process to generate cryopreservable hepatocytes using an iPSC panel of non-alcoholic steatohepatitis (NASH) donors and healthy controls (Gurevich et al., 2020). In drug metabolism research field, team of McCarron developed methods that allow the derivation, proliferation, hepatic differentiation, and extensive characterization of bipotent ductal organoids from NASH patients' irreversibly damaged live (McCarron et al., 2021). Hendriks' team introduces the FatTracer, a CRISPR screening platform designed to identify steatosis modulators and potential targets using $APOB^{-/-}$ and $MTTP^{-/-}$ organoids and identified fatty acid desaturase 2 (FADS2) as a key factor in hepatic steatosis. These organoid models enable the investigation of steatosis causes and drug targets (Hendriks et al., 2023). Kimura et al. devised a pooled human organoid-panel of steatohepatitis to investigate the impact of metabolic status on genotype-phenotype association. "In-a-dish" genotype-phenotype association strategies disentangle the opposing roles of metabolic-associated gene variant functions and offer a rich mechanistic, diagnostic, and therapeutic inference toolbox toward precision hepatology (Kimura et al., 2022). Consequently, organoid-derived fatty liver models are central tools to further study the occurrence, transformation and mechanism of steatosis disease.

2.1.4 Biliary atresia and bile duct defects

Biliary atresia (BA) is characterized by progressive extrahepatic and intrahepatic biliary fibrosis and biliary obstruction. If left untreated, affected patients will eventually develop portal hypertension and liver failure (Lin et al., 2019; Vij and Rela, 2020). BA mainly occurs in neonates, and the etiology is still unclear, which may be related to viral infection, immune damage, environmental and genetic factors (Vij and Rela, 2020). BA is the main indication for pediatric liver transplantation (Zhou et al., 2019). After infection of human cholangiocyte organoids with rotavirus, severe cytopathic changes occurred in the organoid cells, which could partially mimic the development and pathological changes of BA (Chen et al., 2020). Sinobol et al. treated mouse liver ductal organoids with acetaminophen and found that the expression of fibrogenic cytokines and cholangiocyte apoptosis increased, indicating that the organoid model can simulate injury-induced apoptosis of cholangiocytes in BA (Chusilp et al., 2020). Bile duct epithelium organoids were cultured with biliatresone, the cell structure of organoids was destroyed, and the normal apical-basal structure was lost (Lorent et al., 2015). The phenomenon of breakdown in apical-basal polarity was also observed in organoids derived from BA patients or rhesus rotavirus A-infected mice (Babu et al., 2020), which has been confirmed in a recent study (Amarachintha et al., 2022). Cholangiocyte organoids derived from liver biopsies of BA patients showed low expression of developmental and functional markers (cytokeratin 7, EpCAM, transporters aquaporin 1, CFTR), small quantity and misorientation of cilia, a change in the expression pattern of zonula occludens-1 (ZO-1) and increased permeability (Amarachintha et al., 2022). The results above proved that BA patient-derived organoids are excellent models for studying the deficiency of molecular and function in the delayed development of cholangiocytes in BA.

Iatrogenic bile duct injury has become one of the most common causes of benign bile duct defects or strictures with the popularization and broad application of laparoscopic cholecystectomy (Del Vecchio Blanco et al., 2021). Besides, patients who require surgical treatment due to hilar biliary stricture caused by stones can also be observed at present. However, surgical treatment methods for biliary deficit lesions crediting to iatrogenic bile duct injury, BA and hilar biliary stricture have many limitations and disadvantages. Therefore, only biliary-enteric anastomosis can be performed in most patients. Nevertheless, the biliary-enteric anastomosis reconstructs the digestive tract, and the anastomosis fails to function as the Oddis sphincter, which results in a series of postoperative complications such as reflux cholangitis, anastomotic leakage, anastomotic stricture, stone formation, biliary cirrhosis, and even carcinogenesis (Sampaziotis et al., 2017; Matthews et al., 1993; Tocchi et al., 2001; Laukkarinen et al., 2007; Kadaba et al., 2017). As a result, it is still a hot issue to preserve the function of the Oddis sphincter and make the reconstructed bile duct in line with the anatomical structure and physiological function of the normal bile duct. The appearance of organoids provides a certain possibility to preserve the function of the Oddis sphincter. Sampaziotis et al. pioneered the use of human bile duct epithelial organoids to repair the gallbladder and bile duct of mice (Sampaziotis et al., 2017; Tysoe et al., 2019). Another investigation conducted by Sampaziotis' team suggest that organoids have the potential to be utilized for the restoration of human bile ducts (Sampaziotis et al., 2021). Similar findings were described in the study by Roos et al. (2021). These studies provide novel ideas and theoretical bases for the development of treatments for biliary defect diseases.

2.1.5 Primary sclerosing cholangitis

Primary sclerosing cholangitis (PSC) is a cholestatic liver disease of unknown etiology that may be associated with autoimmunity, characterized by biliary inflammation and fibrosis (Reich et al., 2021). Eventually, cholestatic jaundice, cirrhosis, and liver failure develop as the disease progresses. Meanwhile, PSC is also one of the high-risk factors for developing bile duct cancer (Cordes et al., 2019; Dhillon et al., 2019). Soroka et al. collected bile samples from PSC patients using endoscopic retrograde cholangiography and then cultured organoids (Soroka et al., 2019). RNA sequencing showed that PSC-derived organoids changed the expression of 39 genes compared to control organoids. The expression of immune genes (such as HLA-DMA and CCL20) was increased in PSC-derived organoids, and these genes have previously been confirmed to be involved in PSC (Jiang and Karlsen, 2017).

2.1.6 Hepatobiliary cancer

Currently, primary liver cancer, carcinoma of the bile duct and gallbladder are common primary tumors of the hepatobiliary system. Liver tumors are the sixth most prevalent and second most fatal cancer, with increasing incidence in the world (Bray et al., 2018). The current treatment for primary liver cancer is dominated by radical resection, supplemented by arterial chemoembolization, ablation, and sorafenib chemotherapy (Petrowsky et al., 2020; Benson et al., 2021). However, the treatment effect and overall prognosis of liver cancer are poor due to high malignancy and a high recurrence rate after radical resection (Vogel et al., 2018). Cholangiocarcinoma is the second most common malignant tumor of the hepatobiliary system, originating from the bile duct epithelium (Sarcognato et al., 2021). When patients are diagnosed with cholangiocarcinoma, most have unresectable tumors and fail to undergo surgery because of the specific anatomical position, insidious clinical symptoms and early neurovascular invasion and lymph node metastasis (Banales et al., 2020; Zhu, 2015). Although some patients are lucky to be treated with surgery, the tumor is prone to recurrence after the operation and the 5-year survival rate is less than 20% (Kamsa-Ard et al., 2019; Strijker et al., 2019; Cambridge et al., 2021; Cai et al., 2016). Cisplatin plus gemcitabine is the first-line chemotherapy regimen for patients in an advanced stage (Valle et al., 2010; Eckel and Schmid, 2014). Despite the significant effect, the prognosis is still unable to be improved (Koch et al., 2020). Gallbladder cancer, deriving from the gallbladder or the cystic duct, has high malignancy and is prone to metastasis in the early stage. Furthermore, it is usually insensitive to radiotherapy and chemotherapy (Kakaei et al., 2015). Like liver cancer and cholangiocarcinoma, most patients are in the advanced stage when the tumor is found (Schmidt et al., 2019). Therefore, it is considered one of the malignant tumors with poor prognoses in hepatobiliary surgery. Finding personalized and accurate therapy to enhance the outlook of individuals with liver and bile duct tumors continues to be a challenging issue in the medical field. As novel cancer models, the advent of organoids sheds light on this puzzle. At present, oncology research models mainly include human tumor cell lines, mouse models and human tumor xenograft mouse models. However, these models have some unavoidable shortcomings. For example, tumor cell lines lose the genetic heterogeneity of the original tissue during long-term subculture, which fails to reproduce the occurrence, development and metastasis of tumors. In the human tumor xenograft mouse model, it is unavoidable to use murine tumor stroma instead of human tumor stroma with low efficiency, long duration of tumor formation and high cost, limiting this model from being an excellent preclinical model (Bresnahan et al., 2020).

Tumoroids, organoids derived from cancer tissue, have distinct advantages in oncology research. Tumor-derived organoids, like non-tumor epithelial organoids, self-organize via cell-cell and cell-matrix interactions. It has been corroborated that there was a high degree of homology for gene expression profiles between primary carcinoma and liver tumoroids, especially in the expression of hepatocellular carcinoma markers (AFP, GPC3), hepatocyte markers (ALB, TTR, APOA1, APOE), bile duct epithelial markers (EpCAM, KRT19, S100A11) (Broutier et al., 2017). Several studies have demonstrated that biliary tract tumoroids robustly express bile duct epithelial markers (CK19, CK7, EpCAM, S100A6) (Wang et al., 2021; Saito et al., 2019; Maier et al., 2021).

Broutier's team described a novel, near-physiological organoid culture system and extend the 3D culture system to the propagation of primary liver cancer organoids including HCC, cholangiocarcinoma (CC), and combined HCC/CC (Broutier et al., 2017; Broutier et al., 2016). In Nuciforo et al.' study, poorly differentiated hepatic tumors organoids model can also be established derived from needle biopsies (Nuciforo et al., 2018). In 2019, organoids for biliary tract cancer (BTC) were developed from excised tumor tissues (Saito et al., 2019). Similarly, organoids for childhood liver cancers, such as hepatoblastoma (HB), have been developed using a 3D system (Saltsman et al., 2020). The use of surgical specimens from human or murine hepatomas has increasingly become the predominant method for creating liver cancer organoids. While the successful establishment rate of about 30% (Nuciforo et al., 2018), significantly lower than the reported success rates for establishing organoids of pancreatic and colorectal cancer. Thus far, multiple tumoroids have been developed to recapitulate HCC, CC, hepatoblastoma, BTC and combined HCC/CC, which have substantially contributed to liver and bile ducts cancer research for oncologists (Ren et al., 2023). Tumor-derived organoids replicate the histological structure, genomic landscape, gene expression, and tumorigenic potential of the original tumor, offering a novel *in vitro* model for cancer research. Tumoroids preserve the tumor's original diversity and histopathological features both *in vitro* and after xenografting *in vivo*.

The integration of CRISPR/Cas9 and organoid technologies has greatly enhanced the development of tumor models, improving both tumor representation and the accuracy of gene effect predictions. Clevers et al. created human Primary liver cancer (PLC) tumoroids from healthy iPSCs by employing CRISPR-Cas9 to modify the BAP1 gene and furthermore, developed innovative PLC tumoroids by employing CRISPR-Cas9 technology to mutate four genes: NF1, SMAD4, PTEN, and TP53 (Artegiani et al., 2019). CRISPR-Cas9 technology is applicable in liver organoid development due to its genome-modulating capabilities (Artegiani et al., 2019). Artegiani et al. utilized CRISPR-Cas9-mediated homology-independent organoid transgenesis (CRISPR-HOT) technology to tag specific genes and sequences in human

TABLE 1 The examples of hepatobiliary organoids application as disease modeling are listed in the table.

Diseases		Species	Cell source	Expansion medium	Main findings	References
Hereditary disease	Alpha-1 antitrypsin deficiency (AATD)	Human	Bile duct cells	AdDMEM/F12, N2, B27, N-Acetylcysteine, gastrin, EGF, Rspo1, FGF10, HGF, Nicotinamide, A83.01, FSK.	Organoids from A1AT-deficiency patients can be expanded *in vitro* and mimic the *in vivo* pathology	Huch et al. (2015)
		Human	Bile duct cells	AdDMEM/F12, penicillin/streptomycin, Glutamax, Hepes, N-acetylcysteine, Rspo1, nicotinamide, gastrin I, EGF, FGF, HGF, Rho kinase	Liver organoid model recapitulates the key features of Z-AAT deficiency including intracellular aggregation and lower secretion of AAT protein, and lower expression of ALB and APOB.	Gómez-Mariano et al. (2020)
	Alagille syndrome (ALGS)	Human	Bile duct cells	AdDMEM/F12, N2, B27, N-Acetylcysteine, gastrin, EGF, Rspo1, FGF10, HGF, Nicotinamide, A83.01, FSK.	Organoids from an ALGS patient reproduce the structural duct defects present in the biliary tree of these patients	Huch et al. (2015)
		Mice	Bile duct cells	AdDMEM/F12, N2, B27, N-Acetylcysteine, gastrin, EGF, Rspo1, FGF11, HGF, Nicotinamide	Establishment of bile duct–derived organoids from $Jag1^{Ndr/Ndr}$ mice	Andersson et al. (2018)
		Human	iPSCs	RPMI, B27, LDN-193189, CHIR99021, A83-01, EGF, FGF10, HGF.	iPSC-hepatic organoids recapitulate the impaired bile duct formation that is characteristic of ALGS liver pathology, with reduced ability to form bile ducts and impaired regenerative ability	Guan et al. (2017)
	Cystic fibrosis (CF)	Human	iPSCs	William's E medium, nicotinamide, sodium bicarbonate, 2-Phospho-L-ascorbic acid trisodium salt, sodium pyruvate, glucose, Hepes, ITS + premix, dexamethasone, Glutamax, penicillin, streptomycin, EGF.	iPSCs-cholangiocyte-like cells of CF patients model *in vitro* key features of CF-associated cholangiopathy; VX809 rescues the disease phenotype of CF cholangiopathy *in vitro*	Sampaziotis et al. (2015)
		Human	Bile duct cells	AdDMEM/F12, N2, B27, N-Acetylcystein, gastrin, EGF, FGF10, HGF, nicotinamide, A83.01, forskolin, Y27632, R-spondin, Noggin, Wnt, hES cell cloning recovery solution	ECO have cholangiocyte fate differentiation capacity but no potential for hepatocyte-like fate differentiation. ECO derived from a cystic fibrosis patient showed no CFTR channel activity	Verstegen et al. (2020)
	Wilson's disease	Dog	Bile duct cells	AdvDMEM/F12, B27, N2, N-acetylcysteine, gastrin, EGF, R-spondin-1, nicotinamide, HGF, Noggin, Wnt3a, Y-27632, A83-01	Establishment of a long-term canine hepatic organoid culture. Successful gene supplementation in hepatic organoids of COMMD1-deficient dogs restores function	Nantasanti et al. (2015)
			Liver stem cells		The COMMD1-deficient organoid, after restoration of COMMD1 expression, were safely delivered as repeated autologous transplantations via the portal vein	Kruitwagen et al. (2020)
	Wolman disease	Human	iPSCs	AdvDMEM/F12, N2, retinoic acid (RA)/Hepatocyte Culture Medium (HCM), HGF, Dexamethasone, Oncostatin M	Multi-cellular human liver organoids of Wolman disease recapitulated key features of steatohepatitis, and organoid stiffening reflects the fibrosis severity. Severe steatohepatitis was rescued by FXR agonism-mediated reactive oxygen species suppression	Ouchi et al. (2019)

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TABLE 1 (*Continued*) The examples of hepatobiliary organoids application as disease modeling are listed in the table.

Diseases	Species	Cell source	Expansion medium	Main findings	References
Viral hepatitis	Human	iPSCs, HUVECs, BM-MSCs	DMEM/12, GlutaMAX, HEPES, insulin	HBV infection in iPSC-liver organoids could recapitulate virus life cycle and virus induced hepatic dysfunction	Nie et al. (2018)
	Human	Hepatocytes	Ad+++, B27, N2, N-acetyl-L-cysteine, Rspo-1, Wnt3a, nicotinamide, recombinant human gastrin I, EGF, FGF10, HGF, forskolin, A83-01, Noggin, Y27632	Primary *ex vivo* HBV-infection model derived from healthy donor liver organoids after challenge with recombinant virus or HBV-infected patient serum	De Crignis et al. (2021)
Fatty liver	Human	iPSCs	William's E medium, Dexamethasone, SBSB431542, DAPT, OSM.	End-stage hepatocytes derived from non-alcoholic steatohepatitis donors demonstrated spontaneous lipidosis without fatty acid supplementation, recapitulating a feature of NASH hepatocytes *in vivo*	Gurevich et al. (2020)
	Human	Liver stem cells	AdDMEM/F12, Pen/Strep, glutamax, Hepes, B27, NAC, Nicotinamide, R-spondin, N2, FGF-10, HGF, EGF, Gastrin, Forskolin, A83-01, Y27632	Expansion of primary liver stem cells/bipotent ductal organoids derived directly from irreversibly damaged non-alcoholic steatohepatitis patient liver, showing significant upregulation of liver fibrosis and tumor markers, and reduced passaging/growth capacity	McCarron et al. (2021)
Biliary atresia	Mice	Cholangiocytes	Mouse HepatiCult organoid growth medium supplemented with penicillin–streptomycin	First description of cholangiocyte injury in the organoids derived from intrahepatic bile ducts. Fibrogenic response of injured organoids was associated with increased cholangiocyte apoptosis and decreased cholangiocyte proliferation	Chusilp et al. (2020)
	Human	Cholangiocytes	AdDMEM/F12, penicillin/streptomycin, Glutamax, Hepes, B27, N2, N-acetylcysteine, RSPO1, Nicotinamide, Gastrin, EGF, FGF10, HGF, Forskolin, A83-01	Establishment of biliary organoids from liver biopsies of infants with biliary atresia. EGF + FGF2 treatment induced developmental markers, improved cell-cell junction and decreased epithelial permeability	Amarachintha et al. (2022)
Bile duct regeneration	Human	Cholangiocytes	William's E medium, nicotinamide, sodium bicarbonate, 2-phospho-L-ascorbic acid trisodium salt, sodium pyruvate, glucose, HEPES, ITS + premix, dexamethasone, Glutamax, penicillin and streptomycin, EGF, R-spondin and DKK-1	Extrahepatic cholangiocyte organoids can self-organize into bile duct–like tubes after transplantation and can reconstruct the gallbladder wall and repair the biliary epithelium following transplantation into a mouse model of injury	Sampaziotis et al. (2017), Tysoe et al. (2019)
	Human	Cholangiocytes	AdvDMEM/F12, 1M HEPES, L-Ultraglutamine, Primocin, penicillin, streptomycine, N2, B27, N-Acetylcystein, RSPO1, Nicotinamide, Gastrin, EGF, FGF10, HGF, A83-01, Forskolin	Bile-cholangiocyte organoids originate from extrahepatic biliary tissue and are capable of repopulating human extrahepatic bile duct scaffolds. The cells obtain a transcriptomic profile more closely resembling primary cholangiocytes upon repopulation of scaffolds *in vitro*	Roos et al. (2021)

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TABLE 1 (*Continued*) The examples of hepatobiliary organoids application as disease modeling are listed in the table.

Diseases		Species	Cell source	Expansion medium	Main findings	References
Primary sclerosing cholangitis		Human	Cholangiocytes	complete ADF medium, R-spondin, B27, nicotinamide, N-acetyl cysteine, N2, EGF, HGF, FGF10, gastrin, A83-01, forskolin	Bile-derived organoids retain features of cholangiopathies, including the ability to react to inflammatory stimuli by secreting chemokines and propagating immune-reactive phenotype	Soroka et al. (2019)
Hepatobiliary tumor	Hepatobiliary tumor	Human	Tumor cells	AdvDMEM/F12, penicillin/streptomycin, GlutaMAX-I, HEPES, Primocin, B27, N-acetyl-l-cysteine, EGF, FGF10, FGF-basic, HGF, forskolin, A8301, Y27632, Rspo-1, Wnt3a, Noggin	This study delineates heterogeneity of hepatobiliary tumor organoids and proposes that the collaboration of intra-tumoral heterogenic subpopulations renders malignant phenotypes and drug resistance	Zhao et al. (2021)
	Primary liver cancer	Human	Tumor cells	AdvDMEM/F12, Penicillin, Streptomycin, Glutamax, HEPES, B27, N2, N-Acetyl-L-cysteine, Rspo-1, nicotinamide, [Leu15]-Gastrin I, EGF, FGF10, HGF, Forskolin and A83-01	The tumorogenic potential, histological features and metastatic properties of primary liver cancer-derived derived organoids are preserved *in vivo*. Patient-derived organoids are powerful research tool for the drug screening	Broutier et al. (2017), Nuciforo et al. (2018), Saito et al. (2019), Saltsman et al. (2020)
	Extrahepatic cholangiocarcinoma and Gallbladder carcinoma	Human	Tumor cells	AdvDMEM/F12, Penicillin, Streptomycin, Glutamax, HEPES, B27, N2, gastrin, A83-01, Y-27632, EGF, FGF10, R-Spondin1, Noggin, Afamin/Wnt3a CM.	Biliary tract cancer patient-derived organoids show similar histological and genetic characteristics to the corresponding primary tumor tissues. Patient-derived organoids are powerful research tool for the drug screening	Wang et al. (2021), Saito et al. (2019), Ren et al. (2023)

organoids (Artegiani et al., 2020). This technology facilitates organoid research by using fluorescent reporter genes to label and visualize specific molecules. This technology can also induce genetic changes to enhance the development of liver organoids from human fetal cells (Hendriks et al., 2021). CRISPR-HOT technology enables monitoring of cell fate, development, and division, as well as inducing genetic modifications in liver organoids.

Thus, the organoid is a good model to investigate the mechanisms of tumorigenesis, progression, metastasis and recurrence of hepatobiliary cancer. It is also an important tool to predict mutations and develop targets for targeted therapy. Moreover, organoids have the potential to be tools for marker discovery (Broutier et al., 2017). Tumoroids are widely utilized in anti-tumor drug screening as well as precision medicine, and this part is further discussed in the following section. The hepatobiliary organoids application as disease modeling examples are listed in Table 1.

2.2 Biobank

The establishment of organoid biobanks has been facilitated by advancements in the long-term preservation, storage, culturing, and expansion of organoids (Xie et al., 2023). Biobanks facilitate the standardized preservation and collection of PLC tumor samples along with their clinical data. As is mentioned in the previous sections, Broutier et al. established a biobank of PLC tumoroids from seven patients, maintaining the characteristics and expression profiles of the original tumors, including mutations in ARID2, ARID1A, TP53, KRAS, CTNNB1, and WNT1 (Broutier et al., 2017). And Nuciforo and colleagues created an HCC tumoroid biobank that replicated the histopathological and genetic characteristics of original tumors from 38 patients with poorly-differentiated tumors (Nuciforo et al., 2018). Xenograft models demonstrate that PLC tumoroid transplantation in experimental animals induces metastatic traits akin to the original tumors. These data suggest that the tumoroids biobank is suitable for disease modeling, drug testing and validation in PLC and hepatobiliary tumors. Ji' team established a biobank of 65 patient-derived liver cancer organoids, encompassing 44 HCC organoids, 12 intrahepatic cholangiocarcinoma (ICC) organoids, and 4 combined HCC/CC organoids. These organoids comprehensively represent the histological and molecular characteristics of diverse liver cancer types, as determined by multiomics profiling, including genomic, epigenomic, transcriptomic, and proteomic analyses (Ji et al., 2023). Yang et al. established a PLC biobank was with 399 tumor organoids from 144 patients, accurately reflecting the histopathology and genomic characteristics of the original tumors. This biobank is effective for drug sensitivity screening, as demonstrated by *in vivo* models and patient responses (Yang et al., 2024).

2.3 Drug research and precision medicine

Precision medicine aims to enhance disease characterization at the molecular and genomic levels, thereby improving drug screening. Drug screening refers to the screening of new drugs or lead compounds with bioactivity from natural products or synthetic compounds. Due to the advantages described above, lots of studies have utilized organoids as ideal models for drug screening. Patient-specific tumoroids can be established in a short time by culturing tumor specimens obtained by biopsy or surgical resection from the patient (Eckel and Schmid, 2014). The drug screening platform based on patient-specific tumoroids tends to test the sensitivity of the tumor to anticancer drugs in a very short time, providing data support and guidance for individualized treatment.

Broutier et al. tested the sensitivity to 29 anticancer drugs in tumoroids originating from HCC, CC and combined HCC/CC and the results showed that except for CC-2 tumoroid was resistant to all anticancer drugs, and other tumoroids had their respective sensitive drugs (Broutier et al., 2017). Another study used diethylnitrosamine (DEN) to induce liver cancer in mice and generated liver tumoroids from these mice (Cao et al., 2019). Then, they performed drug sensitivity testing and found that 3 samples were sensitive to both sorafenib and regorafenib, 6 were sensitive to only sorafenib, and 4 were not sensitive to both sorafenib and regorafenib. Tissues from different regions of surgical cholangiocarcinoma specimens were obtained to generate 27 tumoroids, which were used to perform drug screening with 129 antitumor drugs (Li et al., 2019). The study showed that during the 129 antitumor drugs, most drugs were only effective against a few tumoroids. But bortezomib, romidepsin, prukamycin, idarubicin, panobinostat, carfilzomibhe and ixazomib were effective against all tumoroids and had moderate or higher killing activity against most tumoroids. Wang et al. constructed 5 gallbladder patient-derived tumoroids (GBC 1–5) and an extrahepatic cholangiocarcinoma (eCCA) patient-derived tumoroid, and found that GBC1 was sensitive to 5-fluorouracil, GBC2 was sensitive to gemcitabine and paclitaxel, GBC3 was sensitive to gemcitabine, GBC4 was sensitive to infigratinib and cisplatin, GBC4 was sensitive to paclitaxel and eCCA was sensitive to gemcitabine (Wang et al., 2021). Additionally, they found that treatment with 10 or 50 μM paclitaxel greatly decreased the growth rate of GBC5 tumoroid, indicating that organoids can be used to identify optimal drug doses. Similarly, a recent study cultured 3 bile duct tumoroids using patient-derived cholangiocarcinoma tissues and transplanted the tumoroids into immunodeficient NSG mice (Maier et al., 2021). Then, the mice formatting tumor successfully were utilized for further *in vivo* drug testing. The experimental results exhibited that tumors in mice treated with gemcitabine stopped growing, while tumors in control mice continued to grow and the response of mice treated with gemcitabine resembled human cholangiocarcinoma patients. Yuan et al. tested 20 targeted drugs approved by the FDA (Food and Drug Administration) that have minimal toxicity to normal gallbladder organoids. The findings indicate that histone deacetylase (HDAC) inhibitors can effectively reduce the growth of gallbladder tumoroids (Yuan et al., 2022).

Consequently, the conclusion can be drawn that different patients have different sensitivities to different chemotherapies or anticancer drugs. The establishment of patient-specific organoid models is able to provide a possibility for drug screening and evaluation of drug efficacy. Meanwhile, organoid xenografts originating from patients exhibited treatment responses analogous to the corresponding patient malignancies, which provide a direct and reliable basis to guide the medication regimen. The organoid is a precision medicine-oriented and efficient preclinical model and has value as an alternative to *in vivo* models.

Other applications of hepatobiliary organoids for medicine research also include drug resistance and toxicity assessment (Zhao et al., 2021; Leung et al., 2020). In addition, hepatobiliary organoids can also be used as a good *in vitro* prediction model of drug hepatotoxicity. In recent years, some research teams have seen the potential of liver organoids and applied them to the assessment of drug metabolic parameters and toxicity, which have been developed to study and predict drug-induced liver injury (Brooks et al., 2021).

While patient-derived organoids (PDOs) are gaining traction in therapeutic screening, various challenges need to be overcome to unlock their full potential. Firstly, the successful establishment of organoids relies on the availability of fresh and viable tissue samples. However, acquiring adequate and high-quality tissue samples for organoid culture is challenging, particularly for some specific tumor types. A further challenge involves the scarcity of patient-derived samples and the ethical issues related to their acquisition. Alternative sources such as minimally invasive procedures or liquid biopsies may be the direction of exploration.

Another major challenge is from the absence of standardized methods for generating and culturing PDOs. Standard methods are essential to ensure the reliability and reproducibility of PDOs as a therapeutic screening model. Differing protocols used by laboratories for their isolation, expansion, and differentiation may lead to the variability in organoid quality and characteristics, which can hinder the comparison of results across studies and the reproducibility of findings in various laboratories.

Organoids should deliver swift outcomes to inform treatment choices within a clinically relevant period. For postoperative adjuvant chemotherapy, 1–3 weeks may be acceptable interval for a drug sensitivity test. However, for neoadjuvant chemotherapy or those advanced tumors, drug screening tests are needed as soon as possible. Efforts are essential to streamline the workflow and minimize the turnaround time for organoid generation and drug sensitivity testing.

Organoids may become contaminated with normal cells during the culturing process. Implementing quality control measures for organoids is crucial prior to drug sensitivity testing. For example, Next-generation sequencing (NGS) is conducted before drug sensitivity testing to verify the presence of key mutations in organoids that influence drug response.

Finally, correlating organoid drug sensitivity testing results with clinical outcomes is crucial to validate its effectiveness in guiding treatment decisions and enhancing patient outcomes. Extensive longitudinal studies involving larger patient cohorts are essential to assess the clinical efficacy and performance of drug sensitivity testing.

2.4 Regenerative medicine

Currently, only liver transplantation can treat various end-stage liver diseases, but the shortage of donors is always a difficulty.

Moreover, as previously described, some patients suffer from bile duct defects due to congenital or acquired causes and there is currently no effective treatment. To overcome this dilemma, an increasing number of researchers valued the value of organoids in regenerative medicine. Yang' team constructed 3D bio-printed hepato-organoids by 3D printing technology and transplanted them into immunodeficient mice with tyrosinemia type I and liver failure (Yang et al., 2021). After being transplanted, the organoids have the ability to develop functional vascular system. Furthermore, the previous section on disease modeling on "Biliary atresia and bile duct defects" mentioned the utilization of human bile duct organoids to restore the gallbladder, bile ducts, and intrahepatic bile ducts in isolated human livers through various studies (Sampaziotis et al., 2017; Tysoe et al., 2019; Sampaziotis et al., 2021).

Autologous organoids may not be feasible for all regenerative medicine applications due to various limitations. Organoid derivation is a time-consuming process, making it unsuitable for patients with acute liver failure who need immediate off-the-shelf regenerative medicine solutions. Second, patient-derived autologous primary organoids might still be influenced by the disease, leading to diminished organ regeneration capacity. Furthermore, access to primary tissue may be unattainable in certain cases, such as cholangiocytes in vanishing bile duct syndrome.

3 Limitations and future perspectives

The past decade has witnessed dramatic progress in organoid technology. Organoids possess distinct advantages as they replicate almost physiological circumstances and maintain parental genetic stability. Disease modeling and drug screening studies can utilize these cells or tissues, which can also be used to treat disorders caused by mutations by reversing the disease-causing mutation. Moreover, organoids exhibit rapid growth and a high rate of success in culture, potentially addressing the issue of low efficiency in forming tumors in patient-derived tumor xenograft models. Nevertheless, the current state of the technology is not fully developed, and numerous obstacles remain that must be surmounted.

Lack of microenvironment sometimes, especially in ASC-derived organoids is the first limitation. Organoid technology serves as an intermediary between cell lines and *in vivo* models, yet it often lacks critical components such as stromal, immune, and vascular endothelial cells needed for thorough modeling. For instance, liver organoids frequently miss hepatocyte zonation and key elements involved in the pathogenesis of metabolic fatty liver disease, including vasculature, immune cells, and neural innervation. This impedes their capability to precisely predict clinical outcomes and prognoses.

As is written in the drug screening section, globally standardized protocols for organoid establishment and quality control are urgently needed. The organoid industry faces challenges due to insufficient standardization, a problem intensified by the swift advancements in engineered organoids. Reproducibility is influenced by batch variations such as patient tissue heterogeneity and the timing and method of iPSC induction, as well as culture conditions like cytokine concentration, matrix gel concentration and composition, and the composition and structure of cells and organoids. Addressing these challenges necessitates collaboration among biomedical scientists, clinicians, and regulatory bodies to standardize organoid technology, thereby easing its transition from research to clinical applications and enabling large-scale organoid production for drug screening.

Relatively higher expenditure compared to traditional models is equally noteworthy. Organoid establishment, maintenance, and passages are costly. The high price of growth factors and medium additives restrict the popularization of organoid culture technology. Only a few laboratories are able to perform organoid culture. To some extent, economic pressures have limited the widespread adoption of organoid technology. Another disadvantage of organoid culture is that it is time consuming, which has also been discussed in the chapter of drug screening.

Furthermore, tissue samples prepared for organoid generation are only small parts of the whole tumor. The higher heterogeneity of tumors questions the reliability of substituting small pieces for whole tumor tissues. Tissue extraction from different sites of the same tumors might better reflect tumor heterogeneity and reliably facilitate cancer translational research.

Organoid technology currently struggles to replicate the complexity of patient-specific immune environments. While coculturing tumoroids with immune cells enhances the modeling of tumor-immune interactions and treatment effects, certain challenges may impede precise modeling and prediction of immunotherapy responses. Different tumor types exhibit unique immune components and varying cell quantities, influencing the immune cell composition in early tumoroid culture and the potential for maintaining and expanding these immune cells. Tumors vary in immune cell composition, with some containing diverse and complex immune cells, while others have immune cells only in the surrounding stroma or lack them entirely. In addition, although preserved immune cells can be maintained initially, they may be lost and diluted over time. Inaccurate modeling of the tumor immune environment limits the utility of organoids in translational and precision medicine.

Vascularization of organoids is still a major challenge. Although implantation of organoids into animals or coculture systems promotes organoid vascularization, these methods only endow organoids with vascular characteristics but not functional perfusion vessels (Shirure et al., 2021). The current microfluidic platform used to establish vascularized organoids is crude and semi-adjustable, and it is affected by multiple factors, including the concentration and composition of cytokines and flow rate. More accurate and flexibly controllable and detectable microfluidic platforms are urgently needed for better vascularization of organoids and accurate prediction of responses to antiangiogenic therapies.

The past decade has witnessed dramatic progress in organoid technology. Organoids faithfully maintain the histological and gene expression characteristics of native tissue, making it the important preclinical models for studying diseases and developing therapies. The use of hepatobiliary organoids technology presents a unique opportunity to investigate the pathophysiological process and disorders of the human hepatobiliary system. These innovative preclinical models hold great potential for future applications. Nevertheless, the organoid method is currently in its early phase and possesses certain limitations. Vascularization of organoids

remains a hotspot in tissue engineering. In the future, how to combine new technologies (CRISPR-HOT, Organ-on-a-chip and so on) with organoid and accelerate translational applications is important.

Author contributions

R-QZ: Writing–original draft, Writing–review and editing. Y-SD: Writing–original draft, Writing–review and editing. FL: Writing–review and editing. S-QY: Writing–review and editing. H-JH: Writing–review and editing. F-YL: Writing–review and editing.

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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.

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Transforming cancer treatment: integrating patient-derived organoids and CRISPR screening for precision medicine

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The persistently high mortality rates associated with cancer underscore the imperative need for innovative, efficacious, and safer therapeutic agents, as well as a more nuanced understanding of tumor biology. Patient-derived organoids (PDOs) have emerged as innovative preclinical models with significant translational potential, capable of accurately recapitulating the structural, functional, and heterogeneous characteristics of primary tumors. When integrated with cutting-edge genomic tools such as CRISPR, PDOs provide a powerful platform for identifying cancer driver genes and novel therapeutic targets. This comprehensive review delves into recent advancements in CRISPR-mediated functional screens leveraging PDOs across diverse cancer types, highlighting their pivotal role in high-throughput functional genomics and tumor microenvironment (TME) modeling. Furthermore, this review highlights the synergistic potential of integrating PDOs with CRISPR screens in cancer immunotherapy, focusing on uncovering immune evasion mechanisms and improving the efficacy of immunotherapeutic approaches. Together, these cutting-edge technologies offer significant promise for advancing precision oncology.

KEYWORDS

patient-derived organoids, CRISPR screening, precision medicine, cancer treatment, immunotherapy

1 Introduction

Cancer is a leading cause of incidence and mortality worldwide (Bray et al., 2024). Despite significant advancements in treatment modalities, including immunotherapy, targeted therapy, and precision medicine (Zeng et al., 2023), challenges such as therapy resistance, tumor heterogeneity, and metastatic progression continue to limit therapeutic efficacy and patient outcomes (Rulten et al., 2023). For instance, resistance to immune checkpoint inhibitors affects up to 60%–70% of patients, while targeted therapies often fail due to the emergence of secondary mutations. This emphasizes the urgent need for *in vitro* models that closely mimick tumors *in vivo* and developing more effective therapeutic strategies to deepen our understanding of cancer biology and to support the development of

precise and personalized treatment strategies. In this context, patient-derived organoids (PDOs) have emerged as a transformative platform, recapitulating tumor complexity and microenvironmental interactions to bridge the gap between traditional models and clinical translation.

In recent years, PDOs have emerged as a pivotal tool in cancer research and therapy. PDOs are three-dimensional (3D) cell culture systems derived from patient tumor tissue that retain the genetic variability and phenotypic diversity characteristic of the primary tumor (Weeber et al., 2017; Verstegen et al., 2025). Unlike traditional two-dimensional (2D) cell cultures and patient-derived xenografts (PDXs), PDOs are able to mimic the tumor microenvironment (TME), enabling the study of interactions between cancer cells and their surroundings and the assessment of therapeutic response in a patient-specific manner (Shi et al., 2024). Moreover, PDOs provide a new *in vitro* model for disease modeling, drug screening and precision medicine in personalized treatment of diseases, while also uncovering therapeutic targets and mechanisms of drug resistance to enhance treatment strategies (Wang Q. et al., 2022).

CRISPR technology has transformed cancer research through precise genetic editing and functional screening, enabling the systematic exploration the roles of driver genes in cancer progression and treatment response (Xue et al., 2020). By creating knockout or activation libraries, it identifies genetic vulnerabilities in cancer cells and reveals interactions between genetic changes and drug responses, supporting targeted therapy development. Moreover, CRISPR screening can provide insight into the interplay between various genetic alterations and therapeutic response, thereby informing the development of targeted therapies (Shi et al., 2023).

The integration of CRISPR screening with PDOs offers a physiologically relevant platform to dissect genetic dependencies within native TME, accelerating the translation of functional genomics insights into precision oncology strategies and personalized treatment. PDOs enable the evaluation of drug sensitivity, while CRISPR-based functional screens identify novel therapeutic targets, paving the way for more effective and individualized therapies (Mundekkad and Cho, 2022). Additionally, this combined approach enhances the understanding of immune evasion mechanisms in tumors, potentially improving immunotherapy strategies. By overcoming drug resistance and refining treatment options, this integration holds promise for better patient outcomes and more precise cancer therapies (Yang et al., 2022).

This review highlights the latest advancements in PDOs, CRISPR screening in cancer research, and their combined potential applications, including studies on oncogenesis, tumor progression and metastasis. It underscores the urgent need for innovative approaches in precision oncology and cancer immunotherapy, emphasizing the transformative role of these technologies in bridging the gap between laboratory research and clinical practice. By advancing personalized cancer treatments, particularly immunotherapies, this integration offers significant promise for improving outcomes for cancer patients.

Abbreviations: PDOs, patient-derived organoids; CRISPR, clustered regularly interspaced short palindromic repeats; 3D, three-dimensional; 2D, two-dimensional; PDXs, patient-derived xenografts; TME, tumor microenvironment; CRC, colorectal cancer; GC, gastric cancer; HCC, hepatocellular carcinoma; CRISPR KO, CRISPR knockout; CRISPRa, CRISPR activation; CRISPRi, CRISPR interference; gRNA, guide RNA; OXL, oxaliplatin; PDAC, pancreatic ductal adenocarcinoma; DOX, adriamycin; TGF-β, transforming growth factor-beta; TKIs, tyrosine kinase inhibitors; IPO11, Importin-11; TSG, tumor suppressor gene; StAR, Stochastic Activation by Recombination.

2 PDOs and their applications

2.1 Cultivation techniques and classification of PDOs

PDOs allow the culture of 3D organ-like structures from patient tissues that retain the genetic and phenotypic characteristics of the primary tumor, providing a more accurate model for studying tumor biology and detecting response to therapy, and representing a major advance in cancer research (Yan et al., 2018). PDOs can be categorized on the basis of their origin, such as tumor-derived organoids from cancerous tissue for tumor-derived organoids, and from healthy tissue for normal tissue-derived organoids. In addition, PDOs can be classified based on their specific applications in research and clinical settings, including drug testing, disease modeling, and personalized medicine (Banerjee and Senapati, 2024).

The establishment of PDOs has revolutionized the field of cancer research by providing a more accurate representation of the tumor microenvironment (TME) than traditional 2D cell cultures. This enhanced fidelity allows for a better understanding of tumor biology, drug response, and the development of targeted therapies (Huang et al., 2024). The establishment of PDOs involves the isolation of cells from a tumor biopsy and their cultivation in a special medium that supports their growth and differentiation. This process not only preserves tumor heterogeneity, but also facilitates the study of the TME, which is critical for understanding cancer progression and treatment resistance (Tong et al., 2024). PDOs have been effectively established from numerous cancer types, such as pancreatic, colorectal, and breast cancers. Moreover, these PDOs have demonstrated their utility in facilitating drug susceptibility assessments and in pinpointing prospective therapeutic targets (Xia et al., 2019), highlighting their potential in the realm of personalized medicine. The culture of PDOs requires meticulous optimization of culture conditions to ensure their viability and function. Various factors, such as the extracellular matrix, the selection of growth factors, and the composition of the culture medium, play a crucial role in the successful establishment and maintenance of PDOs. For example, the use of Matrigel as a scaffold has been shown to enhance structural integrity and growth of organoids, while specific combinations of growth factors can promote differentiation and more closely mimic the *in vivo* environment (Hirt et al., 2022). Recent studies have also explored the use of microfluidic systems to create organoid models on a chip, allowing for better control of the microenvironment and facilitating high-throughput drug screening (Qu et al., 2021).

2.2 Applications of PDOs

The applications of PDOs are immense and impactful, especially in the fields of cancer research and precision medicine. PDOs have helped elucidate the mechanisms of cancer progression and response to therapy, providing insights into tumor heterogeneity and the role of the TME in therapeutic outcomes (Li et al., 2024). PDOs retain the structural integrity and cellular heterogeneity inherent to the primary tumor, including cancer stem cells, differentiated cells, and stromal components, and are essential for studying the mechanisms of tumor behavior and evaluating drug efficacy (Shi et al., 2024). The ability of PDOs to be built up from a wide range of cancer types, including breast, colorectal, pancreatic, gastric, hepatocellular, and other cancers, makes them it a universal model for cancer research (Baskar et al., 2022). Furthermore, to study the complex interactions that occur within tumors, by co-culturing PDOs with stromal or immune cells, the key to how the microenvironment promotes tumor growth, metastasis, and therapeutic resistance has been revealed. For example, studies using PDOs have shown that cancer-associated fibroblasts enhance tumor cell survival and proliferation while also modulating the immune response (Wang Q. et al., 2022; Bulcaen et al., 2024).

PDOs are used to conduct drug screening in addition to studying disease mechanisms, including hereditary, infectious, and malignant diseases (Wang et al., 2023; Han et al., 2024). PDOs allow for the identification of drug sensitivities and resistances against individual patients, and can serve as valuable platforms for drug screening to evaluate therapeutic agents on patient-specific tumor models for efficacy and toxicity (Tang et al., 2022). Studies have shown that drug response in PDOs is strongly correlated with patient prognosis, making them a powerful tool for personalized medicine (Wang et al., 2022b). For example, PDOs have been used to identify effective drug combinations for the treatment of colorectal cancer (CRC), highlighting their potential for optimizing treatment strategies (Huang et al., 2024). Recent studies have also highlighted the ability of PDOs to predict patient response to chemotherapy and targeted therapies, highlighting their potential as a platform for the development of individualized treatment regimens (Zhou Z. et al., 2021; Yu et al., 2022). To conclude, the establishment of patient-derived organoids marks a key advancement in cancer research and treatment, providing a powerful platform for studying tumor biology, optimizing therapeutic strategies, and personalizing patient care. As research continues to evolve, PDOs will play an increasingly integral role in the future of precision oncology.

3 CRISPR screening technology and its applications

3.1 Mechanism and advantages of the CRISPR-Cas9 system

The CRISPR-Cas9 system has revolutionized the field of genetic engineering and molecular biology, providing an efficient and precise method for gene editing (Pacesa et al., 2024). The technology originated in the adaptive immune system of prokaryotes and utilizes short RNA sequences to guide the Cas9 nuclease to specific DNA sequences for targeted modifications (Cong et al., 2013; Mali et al., 2013). CRISPRCas9-medied genome editing to achieve activation/silencing of the target sequence of the target gene is completed in accordance with the three steps of guided RNA target recognition of the target gene sequence, Cas9 nuclease upstream of the target sequence to break the double-strandedness of the target gene, and ligating or repairing the double-strandedness of the break (Asmamaw and Zawdie, 2021). Two of the key components are Cas9 endonuclease and guide RNA (gRNA). gRNA is designed to be complementary to a specific DNA sequence in the target gene, allowing Cas9 proteins to introduce double-stranded breaks at precise locations in the genome, which can be rapidly customized for different target genes. This mechanism enables a variety of gene modifications, including gene knockouts, knock-ins, and transcriptional regulation, which can be used for therapeutic purposes and functional genomics studies (Chen et al., 2019; Li C. et al., 2023).

The versatility of CRISPR-Cas9 has led to its widespread use in different fields, especially in cancer research, where it has been used to explore gene function, build tumor models and identify therapeutic targets (Wang S. W. et al., 2022). Recent advances have introduced derived technologies such as CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa). In addition, CRISPR/Cas9 can target multiple genes at the same time (multiplexing), which is particularly beneficial for the study of complex traits and diseases involving multiple genetic factors (Yu et al., 2022). For example, through the development of the STING-seq method, which integrates genetic association studies, gene editing, and single-cell sequencing, CRISPR screening has been used to establish the link between genetic variation and human health, and to explore genetic predispositions, which provide a basis for understanding the genetic basis of biological processes (Morris et al., 2023). In another study, we integrated multi-omics and CRISPR screening technology to screen chondrocytes for genome-wide knockout and analyze the mechanism of genetic predisposition for height, which highlights the central role of CRISPR screening in analyzing genetic predisposition and exploring key genetic factors, and provides new perspectives and tools for biomedical research (Richard et al., 2025). The low-cost and high-throughput capabilities of CRISPR-Cas9 allow for a wide range of applications in various fields, including agriculture, biotechnology, and medicine (Lu et al., 2021). Despite its advantages, challenges such as off-target effects and delivery methods remain key areas of research to improve the safety and efficacy of CRISPR-based interventions (Zhang et al., 2021).

3.2 Applications of CRISPR screening

Functional CRISPR screens provide valuable insights into the mechanisms of cancer progression and treatment response by enabling high-throughput identification of key genes contributing to tumorigenesis, drug resistance, and metastasis (Chandrasekaran et al., 2022), an approach that reveals novel oncogenic drivers and tumor suppressor genes (Sharma and Giri, 2024). These screens utilize the CRISPR system to introduce targeted gene disruptions throughout the genome, enabling the systematic study of gene function in cancer cells. A significant advantage of CRISPR

screens is their ability to reveal both loss-of-function and gain-of-function mutations, providing a comprehensive understanding of the role of genes in tumorigenesis (Tang, 2024; Wang and Doudna, 2023).

CRISPR screening techniques can be categorized into three main types: CRISPR knockout (CRISPR KO), CRISPRi and CRISPRa. Each method manipulates gene expression in different ways, facilitating the exploration of gene function in different biological environments. For example, CRISPR KO screens are widely used to identify essential genes in cancer cells, helping to reveal pathways leading to tumorigenesis and drug resistance (Zhao et al., 2022).

In the field of immunotherapy, CRISPR screening plays a pivotal role in uncovering critical factors within the tumor microenvironment (TME), enabling the identification of valuable biomarkers to enhance the effectiveness of existing treatments (Cao et al., 2024). For example, CRISPR screening conducted in both comparative 2D cultures and xenografts derived from the same cell line demonstrated the dual role of MEN1 in regulating tumor-microenvironment interactions, offering significant insights for advancing cancer immunotherapy (Su et al., 2024). Furthermore, CRISPR screening techniques have been employed to formulate innovative therapeutic approaches, such as gene therapy and precision medicine. This is achieved by enhancing the genetic underpinnings of diseases and facilitating the creation of targeted treatment strategies (Li Y. R. et al., 2023). A genome-wide CRISPR screening was conducted on CD8-positive T cells to uncover the regulatory factors influencing tumor infiltration in a murine model of triple-negative breast cancer. The findings of this research highlighted established immunotherapeutic targets, including Pdcd1/PD-1 and Hacvr2/Tim-3, while also uncovering novel targets, including Dhx37, that had not been previously reported (Dong et al., 2019). As the field advances, the integration of CRISPR technology with new organoid models is expected to unleash cancer research, greatly improving the treatment outcome of cancer patients.

4 Integration of CRISPR screens with PDOs in cancer research

4.1 Advantages of CRISPR-PDO integration

The integration of PDOs with CRISPR screening offers a transformative approach to cancer research and personalized medicine. The fidelity of PDOs allows for the assessment of drug response and the exploration of therapeutic strategies tailored to individual patients. When combined with CRISPR technology to enable precise gene editing and functional genomics, the use of bioinformatics prediction and screening to identify phenotype-associated genes provides new approaches for reversing drug resistance or combining drugs for cancer treatment and research, with important implications for cancer treatment and research, including identifying key genetic drivers and potential therapeutic targets for cancer, improving the drug screening process, and improving patient prognosis (Figure 1).

A key advantage of utilizing CRISPR screens in PDOs is that it helps to identify phenotype-related genes such as drug resistance genes, cancer driver genes and tumor suppressor genes, unearth potential therapeutic targets, and provide direction for the development of targeted cancer therapies (Ravi et al., 2023). For instance, CRISPR-cas9-mediated gene editing of cancer organoids has been used to validate cancer driver genes and to understand the mechanisms of drug resistance potential targets for therapeutic intervention (Ray and Mukherjee, 2021; Wang H. et al., 2021). Furthermore, the drug screening process can be improved to enhance screening efficiency and accuracy and accelerate anti-cancer drug development. An unbiased genome-wide CRISPR loss-of-function screen in HCC organoids revealing WEE1's vulnerability to oxaliplatin and enhanced therapeutic response to the WEE1 inhibitor adavosertib combination therapy (Jia et al., 2025). By penetrating research into cancer mechanisms and optimizing drug screening, it is expected to find more effective treatment options and improve patient outcomes.

The integration of CRISPR screens with PDOs aids in studying gene-environment interactions in the TME by coculturing organoids with immune cells, revealing how genetic changes in cancer cells affect their microenvironment interactions, crucial for understanding immune evasion and cancer immunotherapy strategies (Takeda, 2021). Moreover, combining CRISPR screens with single-cell RNA sequencing in PDOs has shed light on cancer cell heterogeneity and responses to genetic changes, helping identify subpopulations linked to therapeutic resistance or relapse (Wang et al., 2022d), such as novel targets in breast cancer organoids post-CRISPR knockout (Vishnubalaji and Alajez, 2023).

Together, the integration of CRISPR screens with PDOs represents a powerful approach for dissecting the genetic and molecular underpinnings of cancer. This combined strategy not only enhances our understanding of tumor biology but also has accelerated the discovery of new therapeutic targets and the development of personalized treatment strategies. The subsequent sections delve deeper into specific case studies and the latest advancements in this rapidly evolving field.

4.2 Applications in cancer research

The absence of an *in vitro* tumor model that accurately recapitulates the heterogeneity of human cancers significantly impedes our comprehension of cancer pathogenesis and the assessment of treatment efficacy and toxicity (Marusyk et al., 2020). 3D organoid culture models have demonstrated considerable potential in the representation of human cancers (Kuo and Curtis, 2018; Muthuswamy, 2018; Crespo et al., 2017; Di Modugno et al., 2019). The integration of CRISPR technology aids in the identification of targetable mutations and dysfunctional signaling pathways that are essential for the survival and growth of tumor cells (Stratton et al., 2009). In the following sections, we will focus on these molecular aberrations to identify genes that can inhibit tumor growth and metastasis or reverse drug resistance (Figure 2; Table 1). This approach aims to enhance the efficacy of cancer therapies, reduce drug toxicity, and minimize side effect to heathy cells.

4.2.1 Chemotherapy agents

The identification of tumor targets to reverse drug resistance is critical in enhancing the efficacy of chemotherapy.

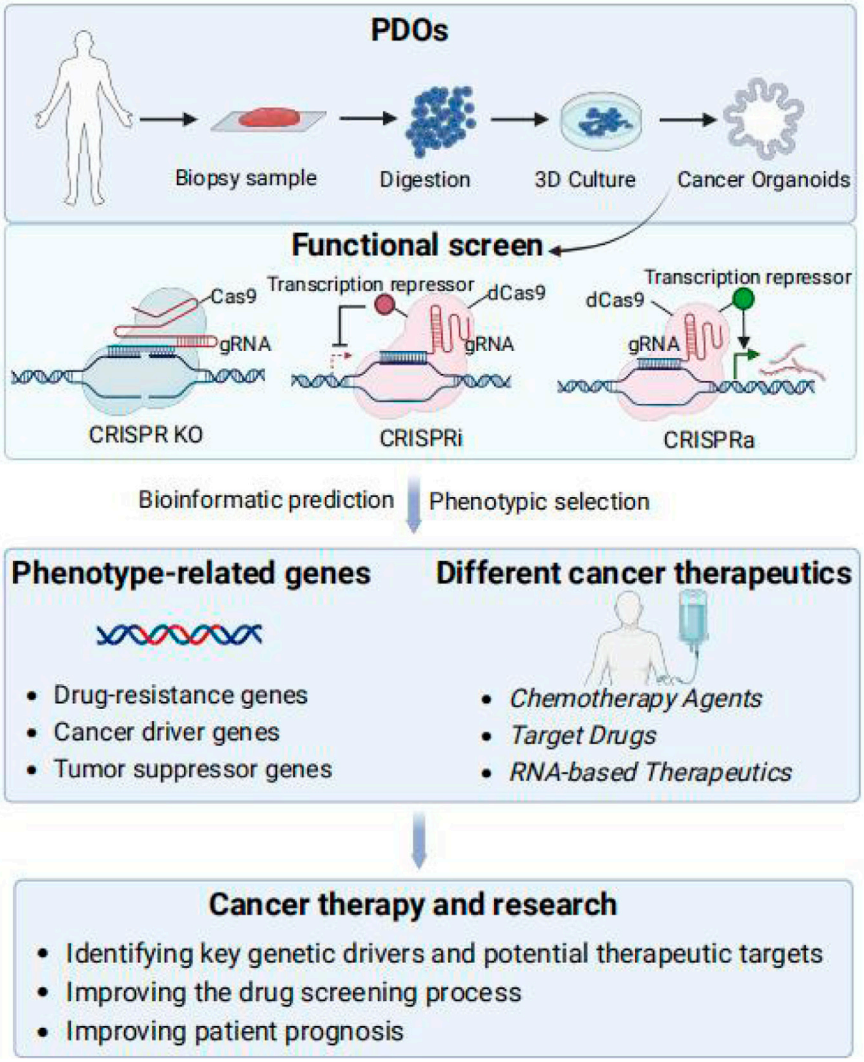

FIGURE 1
Integration of PDOs and functional CRISPR screens. Functional screening in organoids can be realized via CRISPR-Cas screening technologies, including CRISPR KO, CRISPRa, and CRISPRi. After screening for phenotypes, bioinformatics prediction and screening are utilized to identify phenotype-associated genes, drug resistance genes and TSGs as potential therapeutic targets to reverse drug resistance or in combination with drugs provides new methods for cancer treatment and research, including identifying key genetic drivers of cancer and potential therapeutic targets, improving the drug screening process, and enhancing patient prognosis.

Chemotherapeutic agents, including antimetabolites like 5-fluorouracil and anthracyclines, often face challenges due to the development of resistance mechanisms within cancer cells (Feng et al., 2023). By utilizing CRISPR screening in PDOs, genes genes implicated in drug resistance can be systematically knocked out, thus revealing potential targets for therapeutic intervention (Figure 2A).

For instance, the effectiveness of oxaliplatin (OXL), which serves as the primary chemotherapeutic drug for CRC, is frequently hampered by the emergence of drug resistance. In CRC PDOs, CRISPR/Cas9 screening technology has identified MIEF2 as a critical gene associated with resistance. Notably, the diminished expression of MIEF2 correlates with decreased mitochondrial stability and a suppression of apoptosis, thereby influencing the sensitivity of CRC to OXL treatment (Xie et al., 2022). In bladder cancer PDOs, multi-omics analysis and CRISPR screening revealed NPEPPS as a driver of cisplatin resistance, and depletion of NPEPPS was able to re-sensitize drug-resistant cells to cisplatin, whereas overexpression of NPEPPS generated resistance by regulating intracellular drug concentration (Jones et al., 2024). Gemcitabine is the main antimetabolite chemotherapeutic agent for pancreatic ductal adenocarcinoma (PDAC), but many tumors are resistant to it. In PDAC PDOs, a CRISPR-Cas9 screen revealed cytidine deaminases APOBEC3C and APOBEC3D as key genes responsive to gemcitabine that enhance cellular resistance to replication stress by promoting DNA replication fork restart and repair, providing a rational target for improved gemcitabine-based PDAC therapy (Ubhi et al., 2024). Anti-tumor antibiotics such as adriamycin (DOX) are key agents in chemotherapy for osteosarcoma, but the problem of drug resistance severely limits its efficacy. CRISPR screening identified PRKDC as a key determinant of sensitivity to DOX in osteosarcoma, which

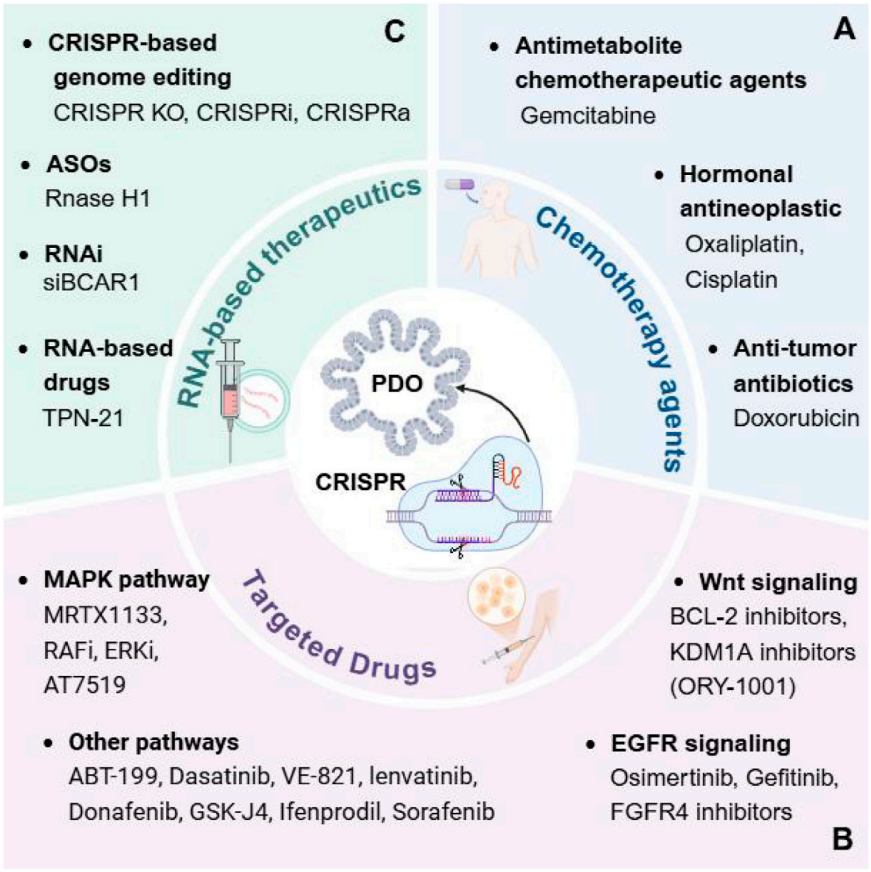

FIGURE 2
Using CRISPR screens in cancer organoids to identify different cancer therapeutics. CRISPR screens for drugs in different cancer PDOs, such as chemotherapy agents **(A)**, targeted drugs **(B)**, and RNA-based therapeutics **(C)**, which in combination with potential therapeutic targets in the screened tumors can reverse resistance or combine them to further improve drug efficacy.

induces chemoresistance by recruiting GDE2 to stabilize GNAS and activate the AKT signaling pathway. Therefore, combining the PRKDC inhibitor AZD7648 with DOX significantly inhibited osteosarcoma growth in organoids, providing a new targeting strategy to improve the efficacy of osteosarcoma chemotherapy (Zhang et al., 2024).

The ability to model these interactions in PDOs allows for a more nuanced understanding of how tumors adapt to therapeutic pressures, paving the way for the development of combination therapies that can effectively overcome drug resistance.

4.2.2 Targeted drugs

Unlike conventional chemotherapeutic agents that indiscriminately affect both cancerous and normal dividing cells, targeted therapies are designed to interfere with specific molecular targets that are involved in the growth, progression, and spread of cancer (Stratton et al., 2009). Cancer cells achieve tumor growth and metastatic dissemination through dysregulation of various signaling pathways, including maintenance of proliferative signaling, evasion of growth inhibitory factors, resistance to cell death, achievement of replicative immortality, induction of angiogenesis, and activation of invasion and metastasis (Hanahan and Weinberg, 2011). The dysregulation of various signaling pathways often underlies these hallmark traits, thereby presenting logical targets for therapeutic intervention. Therefore, the need to improve the efficacy of drugs targeting these signaling pathways is critical (Figure 2B).

One of the most critical pathways associated with cancer is the Wnt/β-catenin signaling pathway, which plays a key role in cell proliferation and differentiation (Clevers and Nusse, 2012). Using CRISPR screen in PDOs to identify resistance genes targeting these pathways may inhibit cancer cell proliferation and induce apoptosis. By CRISPR screening has identified the critical roles of Nf2 and Rasa1 as metastasis suppressor genes in cancer stem cells (CSCs) to regulate invasive features and metastatic ability through Wnt and YAP signaling pathways (Kwon et al., 2023). Furthermore, the result of CRISPR screen in GC PDOs showed that KDM1A inhibitor (ORY-1001) deregulated NDRG1 inhibition, which in turn inhibited Wnt signaling and cell cycle (Mircetic et al., 2023). These findings allow for a more nuanced understanding of how tumors adapt to therapeutic pressures, paving the way for the development of combination therapies that can effectively overcome resistance.

Similarly, dysregulated EGFR signaling, often due to receptor overexpression or mutation, enhances tumorigenesis by promoting uncontrolled cell division, impeding programmed cell death, and facilitating invasion and metastasis (Vaquero et al., 2022). A study suggests that HER2 amplification is associated with resistance to

TABLE 1 Identifying different cancer therapeutics in cancer organoid models using CRISPR screening.

Cancer types	Organoid models	Drug types	Drugs	Functions and mechanisms	References
Colorectal Cancer	OXL-resistant cell lines and organoids	Chemotherapy agents	Oxaliplatin	MIEF2 may serve as a specific biomarker of OXL responsiveness and a potential target for the development of therapeutic approaches to improve chemotherapy efficacy	Xie et al. (2022)
Bladder Cancer	PDOs	Chemotherapy agents	Cisplatin	Intracellular drug transport: NPEPPS affects cisplatin sensitivity by regulating drug concentrations	Jones et al. (2024)
Pancreatic Cancer	PDAC organoids	Chemotherapy agents	Gemcitabine	DNA Replication Stress Resistance: APOBEC3C and APOBEC3D roles identified	Ubhi et al. (2024)
Osteosarcoma	Human organoids	Chemotherapy agents	Doxorubicin	AKT phosphorylation: PRKDC recruited and bound GDE2 to enhance the stability of GNAS, activated AKT phosphorylation and conferred resistance to DOX.	Zhang et al. (2024)
Hepatocellular Carcinoma	HCC PDOs	Chemotherapy agents	Metformin	RAC1 activation, Cell survival: Metformin promotes DOCK1 phosphorylation, leading to resistance	Feng et al. (2022)
Gastric Cancer	Tumorsphere and organoid	Targeted drug	BLC2 inhibitors	Wnt, YAP signaling: NF2 and RASA1 deficiency amplifies these pathways in cancer stem cells	Kwon et al. (2023)
Gastric Cancer	GC PDOs	Targeted drug	KDM1A inhibitors	Wnt signaling, Cell cycle: KDM1A inhibition leads to inhibition of Wnt signaling and G1 cell cycle arrest	Mircetic et al. (2023)
Lung Cancer	Organoids	Targeted drug	Osimertinib, Gefitinib	Hippo pathway: Regulates resistance to EGFR inhibitors	Pfeifer et al. (2024)
Breast Cancer	PDXs and PDOs	Targeted drug	FGFR4 inhibitors	β-catenin/TCF4 signaling pathway, SLC7A11/FPN1 Axis:Involved in glutathione synthesis and Fe2+ efflux, linked to ferroptosis induced by FGFR4 inhibition	Zou et al. (2022)
Breast Cancer	PDOs *in vitro* and cell-derived xenograft or PDXs *in vivo*	Targeted drug	CDK12 inhibitor	PI3K/AKT signaling pathway: Inhibition of CDK12 suppresses this pathway, impacting resistance to lapatinib	Li et al. (2021)
Pancreatic Cancer	PDAC PDXs	Targeted drug	MRTX1133(KRAS G12D inhibitor)	Mechanotransduction, YAP/TAZ expression: ITGB1 influences these pathways to impact KRAS inhibitor efficacy	Kumarasamy et al. (2024)
Pancreatic Cancer	Cell line, organoid, and rat models of PDAC	Targeted drug	RAFi, ERKi	RAF-MEK-ERK cascade: Low-dose vertical inhibition strategy for KRAS mutant PDAC	Ozkan-Dagliyan et al. (2020)
Pancreatic Cancer	3D organoids	Targeted drug	AT7519(CDK1, CDK2, CDK7, CDK9 inhibitor)	CDK network: Hyperactivation linked to mt KRas dependency	Kazi et al. (2021)
Breast Cancer	PDO and PDX models	Targeted drug	ABT-199 (BLC2 inhibitor)	BCL2 pathway: Targeted in combination with CDK4/6 inhibitors for ER + breast cancer	Whittle et al. (2020)
Pancreatic Cancer	PDAC cell lines and organoids	Targeted drug	CDK4/6i, ERKi	CDK4/6, ERK-MAPK, PI3K-AKT-mTOR: Combination therapies targeting these pathways	Goodwin et al. (2023)
Pancreatic Cancer	Human PDAC organoid biobank	Targeted drug	Dasatinib, VE-821	Kinase inhibitor sensitivity: ARID1A mutations associated with sensitivity to dasatinib and VE-821	Hirt et al. (2022)

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TABLE 1 (*Continued*) Identifying different cancer therapeutics in cancer organoid models using CRISPR screening.

Cancer types	Organoid models	Drug types	Drugs	Functions and mechanisms	References
Hepatocellular Carcinoma	Patient-derived primary organoid	Targeted drug	Lenvatinib	Lenvatinib resistance: LAPTM5 could promote intrinsic macroautophagic/autophagic flux by facilitating autolysosome formation	Pan et al. (2023)
Hepatocellular Carcinoma	Animal and PDO models	Targeted drug	Lenvatinib	Mitophagy, antioxidant pathways: LINC01607 regulates these pathways affecting Lenvatinib resistance	Zhang et al. (2023)
Hepatocellular Carcinoma	PDX and PDO models	Targeted drug	Donafenib, GSK-J4	Ferroptosis:Induced by upregulation of HMOX1 and increased intracellular Fe2+ level	Zheng et al. (2023)
Hepatocellular Carcinoma	HCC PDOs and human tumor xenograft models	Targeted drug	Ifenprodil, Sorafenib	Unfolded protein response, WNT signaling, Stemness: Targets of ifenprodil and sorafenib combination	Xu et al. (2021)
Ovarian Carcinoma	RB1-deficient patient HGSC organoids	Targeted drug	Carboplatinum	Cell cycle regulation: CK2 inhibition affects RB family cell cycle regulator p130	Bulanova et al. (2024)
Colorectal Cancer	CRC PDOs	Targeted drug	HDAC inhibitors	H3K9 acetylation and H3K9 dimethylation:Targets of HDAC and EHMT1/2 inhibition	Bamberg et al. (2022)
Colorectal Cancer	Wild-type and APC mutant human intestinal organoids	RNA-based therapeutics	-	TGF-β pathway: Resistance to TGF-β-mediated growth restriction	Ringel et al. (2020)
Colorectal Cancer	Pre-malignant organoids with $APC^{-/-}$;KRASG12D mutations	RNA-based therapeutics	-	TGF-β pathway: Used as a paradigm for screening in organoids	Michels et al. (2020)
Colorectal Cancer	PDOs	RNA-based therapeutics	-	Transplantation of *in vitro* CRISPR-edited cells enables high-throughput and CRISPR-based single-guide rna screening in organoid transplantation to validate cancer cells including colorectal cancer at all stages of its development and treatment	Ray and Mukherjee (2021)
Colorectal Cancer	Primary CRC PDOs	RNA-based therapeutics	-	m6A-dependent oncogenic translation, Wnt signaling: Driven by YTHDF1 and RUVBL1/2	Chen et al. (2024b)
Colorectal Cancer	CRC PDOs	RNA-based therapeutics	-	IPO11 knockout decreased colony formation of CRC cell lines and decreased proliferation of patient-derived CRC organoids	Mis et al. (2020)
Colon Cancer	Colon cancer cells and 3D organoids	RNA-based therapeutics	-	ER stress and ROS production: Linked to stemness and drug resistance in colon cancer	Zhao et al. (2023)
Colorectal Cancer	PDO models	RNA-based therapeutics	-	EMT pathway: ANKRD42 regulates CRC distant metastasis	Liu et al. (2024)
Cholangiocarcinoma	Gene-mutant organoids	RNA-based therapeutics	-	PI3K pathway, Immune microenvironment, Inflammatory responses: Cul3 mutation alters immune microenvironment; other mutations affect various pathways	Feng et al. (2024)
Gastric Cancer	Mouse gastric epithelial organoids	RNA-based therapeutics	-	Wnt signaling: Alk, Bclaf3, Prkra regulate Wnt-driven stem cell-dependent epithelial renewal	Murakami et al. (2021)
Pancreatic Cancer	Tumor organoid cultures from colorectal carcinoma patients	RNA-based therapeutics	-	Wnt signaling: Circuit identified as a vulnerability in RNF43-mutant tumors	Denecke et al. (2023)

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TABLE 1 (*Continued*) Identifying different cancer therapeutics in cancer organoid models using CRISPR screening.

Cancer types	Organoid models	Drug types	Drugs	Functions and mechanisms	References
Breast Cancer	PDOs	RNA-based therapeutics	-	Overexpression of RNaseH1, a ribosomal endonuclease that specifically degrades the r-loop, rescued the reduced clonogenicity caused by TOP1 deletion, suggesting that this vulnerability is driven by aberrant r-loop accumulation	Lin et al. (2023)
Pancreatic Cancer	PDAC organoids	RNA-based therapeutics	-	SRC inhibitor-mediated inhibition of p130Cas phosphorylation impairs MYC transcription via a DOCK1-RAC1-β-catenin-dependent mechanism	Waters et al. (2021)
Pancreatic Cancer	PDO and PDX models	RNA-based therapeutics	-	Targeting aberrantly expressed oncogenic miRNAs and precisely delivering them to tumor cells with the help of nanocomplexes	Gilles et al. (2018)

anti-EGFR therapy (Bertotti et al., 2015). For example, in breast cancer organoids, FGFR4 and CDK12 were identified by CRISPR/Cas9 screening as key factors in anti-HER2 treatment resistance in HER2-positive breast cancers. FGFR4 inhibition enhanced the sensitivity of drug-resistant tumors to HER2 treatment, whereas inhibition of CDK12 restored sensitivity to HER2 tyrosine kinase inhibitors (TKIs) (Zou et al., 2022; Li et al., 2021). These findings provide new therapeutic targets to overcome drug resistance in HER2-positive breast cancer.

The RAS/RAF/MEK/ERK signaling pathway, frequently referred to as the MAPK pathway, constitutes a crucial cascade that orchestrates processes such as cellular proliferation, survival, and differentiation. Among the various genetic modifications observed in human malignancies, mutations in the KRAS gene are recognized as some of the most prevalent alterations (Takeda et al., 2019). Recent advances have led to the development of covalent inhibitors targeting specific KRAS mutations, e.g., studies have revealed the potential of MRTX1133 targeting KRAS G12D in PDAC therapy, and a CRISPR screen identified ITGB1 as a key target for enhancing the response to MRTX1133 therapy by regulating mechanotransduction signaling and YAP/TAZ expression (Kumarasamy et al., 2024).

In addition to these three common pathways, there are some drugs that target other pathways. CDK4/6 inhibitors significantly prolong tumor response in patients with metastatic estrogen receptor-positive (ER+) breast cancer, but recurrence is almost inevitable (Spring et al., 2020). A CRISPR/Cas9 screen showed that ABT-199 is a potent and selective BCL2 inhibitor that in combination with CDK4/6 inhibitors has potential for the treatment of ER + breast cancer (Whittle et al., 2020). VEGF is a potent angiogenic factor that is inhibited by drugs targeting the VEGF receptor such as signaling tyrosine kinase inhibitors (e.g., sunitinib and sorafenib) (Lee et al., 2015). However, drug resistance limits clinical efficacy. A CRISPR-Cas9 screen identified that the combination of the NMDAR antagonist Ifenprodil with sorafenib significantly increased antitumor activity in HCC PDOs (Xu et al., 2021). PARP inhibitors exploit synthetic lethality by targeting the DNA repair pathway. A CRISPR-Cas9 screen revealed a significant increase in the antitumor activity of the NMDAR antagonist ifenprodil in RB1-deficient HGSC and TNBC-like organs, CK2 inhibitors were able to enhance sensitivity to PARP inhibitors, providing a novel strategy to overcome therapeutic resistance in these cancers (Bulanova et al., 2024). The study also identified countless new targets and pathways with tumor therapeutic potential. These include targeting the TME, modulating the cancer epigenome, inhibiting the proteasome and interfering with cancer metabolism. Targeting specific molecular aberrations, such as by CRISPR-Cas9 screening, we found that knockdown of EHMT1/2 significantly enhanced the anti-proliferative effect of HDAC inhibitors in CRC, suggesting that EHMT1/2 is a potential epigenetic therapeutic target (Bamberg et al., 2022).

On the basis of the PDOs model, the use of CRISPR screening not only aids in the identification of novel therapeutic targets but also informs the design of combination therapies that can preemptively address potential resistance mechanisms, ultimately enhancing the efficacy of targeted treatments in clinical settings. By integrating these methodologies, researchers can better navigate the complexities of tumor biology and improve therapeutic strategies for patients facing drug-resistant cancers.

4.2.3 RNA-based therapeutics

Compared to conventional protein-targeted and DNA-based medicines, RNA-based therapeutics hold great promise due to their distinct physicochemical and physiological properties (Crooke et al., 2018). RNA-based therapeutics represent a rapidly evolving frontier in the treatment of various diseases, particularly in oncology, cardiovascular diseases, and genetic disorders. These therapies utilize RNA molecules to modulate gene expression and protein synthesis, providing a targeted approach to disease management (Figure 2C).

ASOs are short, single-stranded oligonucleotides that can pair with specific RNA complementary bases to reduce, restore or modify protein expression by can alter RNA (Crooke et al., 2021). One of the degradation mechanisms mediated by RNaseH1 is also known as enzymatic RNA degradation (Liang et al., 2017). A genome-wide

CRISPR knockdown screen revealed TOP1 as a potential target for synthetic lethality in MYC-dysregulated breast cancer cells. Overexpression of RNaseH1 reversed the decrease in clone-forming ability induced by TOP1 deletion, highlighting its importance in regulating aberrant r-loop accumulation and DNA repair. It may also affect gene expression and DNA repair processes by regulating RNA-DNA hybrids, which may have an impact on cancer cell survival and proliferation (Lin et al., 2023).

RNAi induces double-stranded RNA degradation of specific RNA targets, providing an intrinsic defense mechanism against invading viruses and transcription factors (Kim and Rossi, 2007). SiRNAs can induce RNAi in mammalian cells (Castanotto and Rossi, 2009), thereby establishing this targeted gene silencing mechanism as a robust experimental approach for functional genomics research. BCAR1 was identified as a potential therapeutic target through the use of siRNA screening and CRISPR-Cas9 genomic screening in pancreatic cancer-like organs. Inhibition of BCAR1 enhanced the sensitivity of pancreatic cancer cells to ERK inhibitors, while the combination of SRC inhibitors and microtubule protein inhibitors synergistically reduced MYC protein levels and effectively inhibited the growth of KRAS-mutant pancreatic cancers (Waters et al., 2021).

The prokaryote-derived CRISPR-Cas system has been widely used in mammalian cells and organisms for precise genome sequence editing, which is an efficient genome editing (Doudna and Charpentier, 2014). SgRNAs play the role of guides in the CRISPR-Cas9 system, and by designing specific sgRNA sequences, they are able to guide the Cas9 proteins to specific sites in target DNA sequences, enabling the cleavage and editing of specific locations in the genome (Michels et al., 2020). One study systematically demonstrates the utility of sgRNA-based screening platforms in elucidating TGF-β signaling components that drive colorectal cancer (CRC) progression, highlighting their potential as therapeutic targets (Ringel et al., 2020).

While CRISPR-based therapeutics hold the potential to precisely target virtually any genomic locus, their clinical translation remains contingent on the development of delivery systems capable of ensuring cell-specific uptake while circumventing unintended immune activation (Madigan et al., 2023). Recent advancements in nanotechnology have significantly enhanced the delivery systems for RNA-based drugs, improving their stability and bioavailability (Ren et al., 2012). The therapeutic potential of a nanomedicine, TPN-21, was first demonstrated to reduce tumor cell growth and survival in individual patients with PDO avatars. By utilizing PDO as a rapid screen for TPN-21, RNA-based oligonucleotides packaged in a TPN targeting a specific PDAC tumor receptor demonstrated that TPN-21 strongly inhibits PDO growth even in the presence of gemcitabine failure (Gilles et al., 2018).

However, the most significant obstacle preventing the widespread usage of RNA-based approaches is the difficulty of efficiently delivering such drugs to target organs and tissues apart from the liver. Most delivery methods for these RNA-based therapeutics can be categorized as the addition of targeting moieties, the encapsulation of RNAs into lipid-based nanoparticles, and direct delivery into the target organ without extensive modification. In addition, off-target binding (Kamola et al., 2015), sequence-induced toxicity, and oversaturation of the endogenous RNA processing pathway affect the effectiveness of RNA-based approaches (Grimm et al., 2006). Despite the inherent limitations of current RNA-based therapeutic platforms, ongoing mechanistic studies, including exogenous RNA delivery strategies and small molecule-mediated RNA targeting approaches, promise to enable the systematic development of next-generation RNA-targeted drug therapeutics with improved clinical translational potential.

5 Therapeutic implications of PDOs and CRISPR screens in cancer immunotherapy

Cancer immunotherapy has emerged as a revolutionary approach in oncology, harnessing the body's immune system to fight malignancies. The promise of immunotherapy is immense, as evidenced by the remarkable advances in immune checkpoint inhibitors and CAR-T cell therapy, which have shown significant efficacy in a variety of cancers, including melanoma and hematologic malignancies (Ni et al., 2023). Despite these advancements, significant challenges persist. Many patients exhibit primary or acquired resistance to these treatments, limiting their effectiveness (Bockamp et al., 2020). In addition, an immunosuppressive barrier often exists in the TME, preventing immune cell infiltration and activity (Bader et al., 2020). Tumor heterogeneity further complicates treatment, as different tumor types and even subtypes within a single tumor may respond differently to immunotherapeutic agents. In addition, the risk of immune-related adverse events poses a significant problem that requires a careful balance between efficacy and safety (Meier et al., 2024). Addressing these challenges is critical for the successful incorporation of immunotherapy into standard cancer care, highlighting the need for innovative strategies to improve outcomes.

The integration of PDOs with CRISPR technology represents a groundbreaking innovation in cancer immunotherapy research. This approach not only deepens our understanding of tumor biology but also enables personalized medicine by allowing the screening of patient-specific responses to a broad range of immunotherapeutic agents. Additionally, the capability to model the TME in PDOs offers critical insights into mechanisms of immune evasion, paving the way for the development of more effective immunotherapies that can overcome these barriers.

The integration of CRISPR-Cas9 technology with PDOs screen for phenotype-related genes that can target tumor cell death in cancer immunotherapy, including inhibition of cell proliferation and induction of cell death (Figure 3A). Importin-11 (IPO11) was identified as an essential factor for β-catenin–mediated transcription in APC-mutant CRC cells by a genome-wide CRISPR screen. IPO11 knockdown reduced colony formation in CRC cell lines and reduced proliferation of patient-derived CRC-like organs, revealing that IPO11 promotes β-catenin nuclear import that cancer therapy by reducing cell proliferation (Mis et al., 2020). Similarly, in HCC, the strategic application of CRISPR-Cas9 screening has significantly advanced the identification of synergistic drug combinations that enhance cell death induction. For instance, the combination of donafenib with GSK-J4 has been shown to promote ferroptosis in liver cancer models, a process driven by the synergistic upregulation of HMOX1 expression

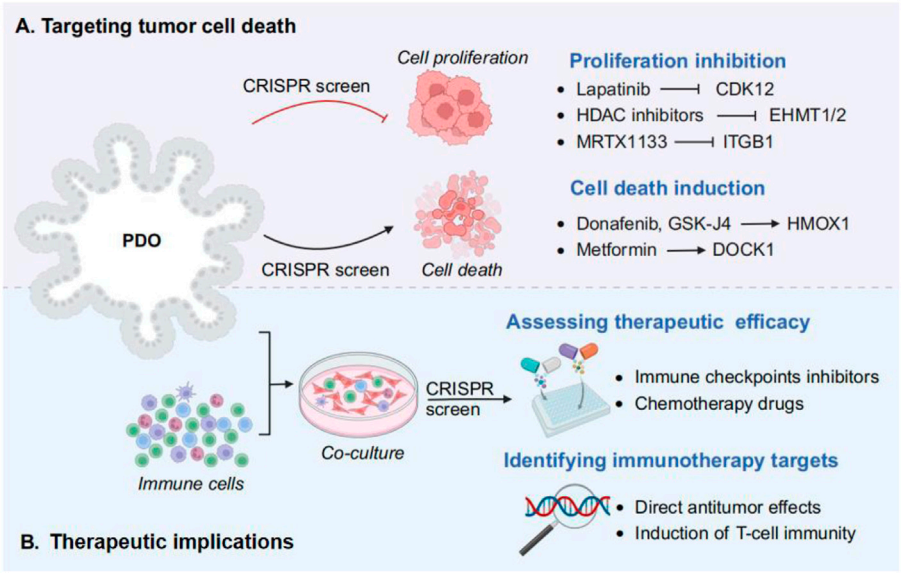

FIGURE 3
Using CRISPR screens in PDOs for cancer treatment strategies. **(A)** Targeting tumor cell death. The role of phenotype related genes and drugs in tumor cell death mechanism, including inhibition of cell proliferation (e.g., CDK12, EHMT1/2, and ITGB1), and induction of cell death (e.g., HMOX1 and DOCK1). **(B)** Therapeutic implications. By incorporating immune cells into PDO culture, followed by CRISPR screening to assess the efficacy of drug therapy and identify the immunotherapy targets.

alongside increased intracellular iron levels (Zheng et al., 2023). These developments signify a paradigm shift towards personalized and precision oncology, wherein genetic insights are leveraged to design tailored therapeutic strategies that effectively inhibit unchecked cancer cell proliferation, revolutionizing the landscape of cancer treatment.

The amalgamation of PDOs with CRISPR-Cas9 technology has also opened new avenues by assessing therapeutic efficacy and identifying immunotherapy targets in cancer therapy (Figure 3B). Compared with traditional 2D cultures or animal models, PDOs provide a more accurate representation of the TME, making them invaluable for preclinical testing of immunotherapeutic agents. By incorporating immune cells into PDO cultures, researchers can better mimic the interactions between cancer cells and the immune system, thereby enhancing the predictive power of these models for immunotherapy responses (Grönholm et al., 2021). For example, studies have demonstrated that systematically dissect PDA intrinsic mechanisms of immune evasion by CRISPR screening in PDA organiods, and identify Vps4b and Rnf31 as essential factors required for escaping CD8 T cell killing (Frey et al., 2022). Therefore, PDOs can be used to evaluate the efficacy of immune-related targets and various immunotherapeutic approaches, thereby shedding light on the mechanisms underlying immune evasion and resistance (Xiang et al., 2024).

CRISPR-Cas9 technology further enhances the utility of PDOs by enabling precise genetic modifications. This allows for the systematic investigation of gene functions and the identification of novel therapeutic targets. In recent years, the gene editing technology of CRISPR-Cas9 has been used in clinical trials of human immunotherapy (Sun et al., 2022). Immunotherapy based on gene editing technology mainly modifies T cells to recognize tumor antigens and increase anti-tumor activity (Osborn et al., 2016). By knocking out specific genes in PDOs, researchers can study the effects of these genetic alterations on tumor-immune interactions and identify potential vulnerabilities that can be targeted by immunotherapies (Sun et al., 2023). CheckCell-2 (NCT05566223), a CRISPR/Cas9-based CISH gene therapy, knockdown of CISH in CD8 (+) T cells improves the efficacy of cancer immunotherapy. CD70 effectively regulates T cells and promotes immunosuppression in the TME, a target for immunotherapy. CTX131 (NCT05795595) and CTX130 (NCT04438083), immunotherapies targeting allogeneic CD70 CAR T cells, enhance the efficacy of CAR T cell therapy for relapsed and refractory solid tumors (Dewulf et al., 2023; Feng et al., 2020). Moreover, the combination of PDOs and CRISPR-Cas9 technology has facilitated the development of personalized cancer immunotherapy approaches. By creating organoids from individual patients' tumors and using CRISPR to introduce specific genetic modifications, researchers can tailor immunotherapeutic strategies to the unique genetic and immunological landscape of each patient's cancer (LaFleur and Sharpe, 2022). This personalized approach not only improves the efficacy of immunotherapies but also reduces the risk of adverse effects by targeting the specific pathways involved in each patient's tumor (Dao et al., 2022).

In summary, the integration of PDOs with CRISPR-Cas9 technology represents a powerful tool for advancing cancer immunotherapy. By providing a more accurate and personalized model of the TME, this approach facilitates the discovery of novel therapeutic targets, the assessment of immunotherapeutic agents, and the formulation of personalized treatment strategies. With advances in novel technologies, it offers significant potential to increase the effectiveness of cancer immunotherapy and bring us closer to the goal of precision oncology (Bonaventura et al., 2022).

6 Conclusion and future perspectives

In the rapidly evolving landscape of cancer research, the integration of PDOs and functional CRISPR screens has emerged as a transformative approach, offering unprecedented insights into tumor biology and therapeutic vulnerabilities. This review emphasizes the integration of PDOs and CRISPR screening in tumor therapy and their potential therapeutic applications. Additionally, we highlight common applications of both technologies in tumor progression to reverse drug resistance or perform combination therapy by targeting multiple signaling pathways associated with cell proliferation, apoptosis, and metastasis through screened potential targets. Importantly, combined PDOs and CRISPR screening can explore new potential therapeutic targets for cancer as well as new targeted drugs for more precise individualized medicine. The combined use of PDOs and CRISPR screening may provide important hints for understanding the biological functions of cancer and provide new clinical value for individualized cancer medicine.

While combining organoid and CRISPR screening has shown attractive breakthroughs and prospects, the field is yet to conquer certain challenges and limitations. Firstly, current techniques for PDOs culture are inherently uncontrollable and non-repeatable due to several non-standardized aspects of cancer tissue source and subsequent processing, media formulation, and animal-derived 3D matrices. For example, culture media containing multiple growth factors and nutrients because of the inclusion of some of these components as purified recombinant proteins may also be limited by poor solubility and insufficient long-term storage stability, resulting in diminished protein activity. Moreover, the use of animal-derived sera introduces heterologous components that may adversely affect embedded organ models and limit human-specific immunological studies, as well as the risk of animal-derived, bacterial, or viral infections (LeSavage et al., 2022). To solve this difficulty, standardized media formulations can be developed to reduce heterogeneity and non-replicability of components. Secondly, despite the ability of organoids to mimic many features of neural development, the lack of some important physiological processes, including vascularization and transmission of signal mediators remains a key issue. For example, brain organoids are prone to necrosis in the central part due to the lack of blood vessels, which affects their normal development and neuronal migration (Qian et al., 2019). The microfluidic chip can simulate dynamic perfusion, combining microfluidic technology with 3D-like blood vessels, supplemented with perfusion functions on the basis of having a vascular structure to fully simulate real blood perfusion (Bhatia and Ingber, 2014; Kistemaker et al., 2025; Birtele et al., 2024). Thirdly, potential gene targets or drug targets obtained through PDOs and CRISPR screening have clinical translation uncertainties. They may not have the expected therapeutic effect *in vivo*, and may even produce adverse reactions, increasing the risk and cost of clinical translation. Their effectiveness and safety in clinical treatment need further validation. The assessment of the safety of gene therapy can be briefly summarized in two aspects, assessment of genomic integrity (Pacesa et al., 2022; Jones et al., 2021) and identification of off-target editing activity (Cameron et al., 2023; Newton et al., 2019). Currently, development of new genetic screening techniques, such as CRISPR-StAR (Stochastic Activation by Recombination) technology can significantly improve the reliability and resolution of data, reducing the risks and costs of clinical translation (Uijttewaal et al., 2024).

Despite current challenges and limitations, combining PDOs and CRISPR screening with other technologies can further develop precision medicine and enhance individualized care. Artificial intelligence (AI)-driven drug discovery employs machine learning and deep neural networks to predict drug-target interactions and optimize molecular designs, overcoming cost and efficiency barriers of traditional methods (Li and Zhang, 2013; Hopkins, 2008; Krishnamoorthy et al., 2024; Lucas et al., 2024), while network pharmacology maps drug-polypharmacology networks to identify multi-target intervention strategies, particularly in oncology, by analyzing pathway interconnectivity and enabling mechanism-based therapeutic repositioning (Both et al., 2023; Reyna et al., 2022; Wang X. et al., 2021; Zhao et al., 2024; Zhou W. et al., 2021). Through the combination of these two technologies, off-target effects are systematically predicted at the preclinical stage and drug combinations are rationally designed to circumvent resistance mechanisms in tumor therapy (Mayer et al., 2024). AI-enhanced analysis of PDOs enables real-time therapy optimization (Co et al., 2024), while CRISPR-AI integration accelerates multi-target discovery through machine learning-prioritized gene editing (Jakavonytė-Staškuvienė and Mereckaitė-Kušleikė, 2023). Network pharmacology further empowers this framework by mapping drug-pathway polypharmacology, establishing a tripartite precision medicine platform in PDOs that synergizes clinical data mining, genetic target identification, and multi-target therapeutic design (Joshi et al., 2024). Therefore, AI and integrated network pharmacology in the fusion of PDOs and CRISPR screening techniques is expected to improve the accuracy and effectiveness of precision medicine. Moreover, CRISPR-engineered PDOs enable complex mutation modeling and scalable disease interrogation (Geurts and Clevers, 2023), while integration with single-cell multiomics, RNA-targeted editing, and immune-competent coculture systems provides unprecedented resolution in mapping tumor-immune dynamics (Ferreira da Silva et al., 2024; Chen Y. et al., 2024), synergistically advancing personalized therapeutic discovery.

In conclusion, integrating PDOs with functional CRISPR screening offers transformative potential for cancer research and therapy. These innovative approaches can expedite the identification of novel therapeutic targets, refine drug screening methodologies, and significantly improve patient outcomes. To unlock their full potential in the fight against cancer, ongoing interdisciplinary collaboration and technological advancements will be indispensable. Future research should focus on validating these targets in clinical trials, exploring combination therapies, or investigating their role in other cancer types to further translate these findings into effective treatments. By addressing these issues, we hope to bridge the gap between basic research and clinical applications, and ultimately contribute to the development of more precise and effective cancer therapies.

Author contributions

ZZ: Writing–original draft. JS: Writing–original draft. PH: Writing–review and editing. YH: Project administration, Writing–review and editing. ZM: Funding acquisition, Project administration, Writing–review and editing. LW: Project administration, Supervision, Writing–review and editing.

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Celastrol reduces cisplatin-induced nephrotoxicity by downregulating SNORD3A level in kidney organoids derived from human iPSCs

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Background: Celastrol, an active ingredient derived from *Tripterygium wilfordii Hook F*, has shown therapeutic potential for various kidney renal diseases. The kidney protective activity of celastrol is mainly exerted through anti-inflammatory, and antioxidant effects. However, celastrol causes dose-dependent kidney toxicity, which results in increased risks of mortality among patients. This study aimed to develop a kidney organoid-based prediction system to assess the safety and efficacy of celastrol in reducing cisplatin-induced nephrotoxicity.

Methods: We investigated the ability of celastrol to reduce cisplatin-induced nephrotoxicity using kidney organoids. Kidney organoids were cultured and characterized, exhibiting renal tubular and glomerular structures and expressing specific kidney markers such as NPHS1, CD31, LTL, and SLC12A1. Data were obtained from *in vitro* experiments in which kidney organoids were exposed to therapeutically relevant concentrations or a toxic dosing profile of cisplatin and celastrol, to assess their impact on cell viability using flow cytometry and Acridine Orange/Propidium Iodide (AO/PI) staining. In addition, RNA-seq analyses were performed to determine the mechanisms of celastrol function in the kidney.

Results: Kidney organoids exposed to 50 μM cisplatin showed significantly increased cell death (only 0.37% cells with normal cell structure), whereas celastrol under 5 μM (56% cells with normal cell structure) showed significantly less nephrotoxicity than cisplatin. The protective effects of celastrol against cisplatin-induced nephrotoxicity were further investigated by treating the organoids with both compounds. The results demonstrated that 2 μM celastrol reduced cisplatin-induced nephrotoxicity by downregulating SNORD3A and HIST1H3A gene levels.

Conclusion: This study highlights the potential of celastrol as a protective compound against cisplatin-induced kidney damage and emphasizes the

importance of using advanced models, such as iPSC-derived kidney organoids, to predict therapeutic effect and nephrotoxic concentrations of novel drugs.

KEYWORDS

celastrol, kidney organoids, nephrotoxicity, cisplatin, SNORD3A

Introduction

Chinese medicine has been used clinically to treat various diseases for thousands of years. Its acceptance has grown in recent years because of its therapeutic effectiveness (Xin Luan et al., 2020). As society has progressed and developed, it has gained popularity around the world and is now generally regarded as a supplement and an alternative therapy in many countries (Zhang et al., 2021). According to available data, the traditional Chinese medicine business is enormous, with Chinese herbs exported to more than 175 countries and territories, including Japan, South Korea, India, Germany, the Netherlands, European countries, and the United States (Yang et al., 2018).

Many Chinese herbal medicines have good therapeutic effects but can cause adverse effects such as nephrotoxicity (Shi et al., 2020). For example, several contain nephrotoxic components, such as aristolochic acids and other alkaloids, that can lead to kidney problems such as acute kidney injury and chronic kidney disease. Celastrol, derived from *Tripterygium wilfordii* Hook F, has anti-inflammatory and antitumor properties (Boran et al., 2019), treats renal diseases, and has nephrotoxic effects (Wu et al., 2021). Certain concentrations of celastrol have been found to be toxic, whereas lower doses have demonstrated a protective effect against kidney damage. Understanding the dual effects and dose-dependent toxicity of celastrol is essential for its safe and effective use in clinical settings. According to Jiang et al. (Jiang et al., 2013), high concentrations of celastrol (>1.0 μM) caused G2/M arrest and apoptosis in Huh7 cells by activating caspase3/7, whereas low concentrations (<1.0 μM) exhibited no evident effects. Low-concentration celastrol had substantial combinatorial effects with Phytohemagglutinin (PHA) on Huh7 cells and Huh7 xenografts, inhibiting proliferation and migration and inducing apoptosis. Zhang et al. (Jianhe) discovered that large doses of it worsened renal damage in model rats. Although it causes dose-dependent renal toxicity in normal rats, it also exerts a protective effect on the pathology of kidney damage at certain doses. It is vital to correctly comprehend the "two-way effect" of celastrol's protection and damage, as well as its "dose-effect (toxicity) relationship". Thus, greater attention should be directed toward the judicious use of celastrol and related preparations (Lianqi).

Nephrotoxicity, the second most common type of drug-induced damage in critically ill patients, accounts for approximately 25% of kidney failures in this group (Astashkina et al., 2012). Traditional animal models, which often use rats, mice, and rabbits, have limitations in accurately predicting human responses owing to interspecies differences and high costs. *In vitro* models utilizing proximal renal tubular cells, such as human and canine epithelial cell lines (ZHUANG Yan-shuang et al.), are commonly used for early nephrotoxicity screening. However, these models lack a three-dimensional tissue structure and inter-organ interactions, failing to fully represent the effects of drugs on kidney tissue. Human-derived *in vitro* models, particularly 3D kidney organoids from induced pluripotent stem cells (iPSCs), have been used (Wu et al., 2023). These organoids mimic the physical and functional aspects of human kidney tissues and include multiple cell types (Matsui and Shinozawa, 2021; Chen et al., 2021). Unlike 2D cultures, organoids can sustain specific cell phenotypes for extended periods, making them more suitable for high-throughput drug screening (Khoshdel Rad et al., 2020; Kim et al., 2020). Ueno et al. (2022) showed that kidney organoids are effective for nephrotoxicity assessment, offering comprehensive insights into the underlying mechanisms. Thus, iPSC-derived kidney organoids are a valuable platform for modelling organogenesis and evaluating human nephrotoxicity (Nauryzgaliyeva et al., 2023; Freedman et al., 2015).

Platinum-based chemotherapeutics, such as cisplatin, have been pivotal in cancer treatment for decades, particularly against lung, ovarian, brain, and breast cancers. Despite its effectiveness, its side effects (mainly nephrotoxicity) limit its clinical use (Shi et al., 2022). Several studies have found that celastrol can ameliorate cisplatin-induced nephrotoxicity via oxidative stress (Yu et al., 2018). This study focuses on develop the *in vitro* predictive assays using iPSC-derived kidney organoids, which more closely resemble the structure of adult kidney tissue, could help predict the potential protective effects of celastrol treatment against cisplatin-induced nephrotoxicity more accurately in humans.

Materials and methods

Human iPSCs culture

Human iPSCs were obtained from CAS key laboratory of regenerative biology. The cells were cultured on growth factor-reduced Matrigel (354277, Corning Life Sciences, Kennebunk, ME, United States)-coated 6-well plates (0.013 mg/cm^2) in mTeSR medium (85875, Stemcell Technologies, Vancouver, BC, Canada) and cells were subcultured every 3–4 days at a ratio of 1:6.

Self-organization of kidney organoids

The culture of iPSC-derived kidney organoids in this study was initiated according to the method proposed by Przepiorski et al. (2018). Before differentiation, iPSCs were maintained on a 10-cm Matrigel-coated cell culture dish to approximately 75% confluence. On day 0, embryoid bodies were generated by detaching colonies into single cells with1 mg/mL dispase (17105041, GIBCO, Grand Island, NY, United States) and then cells were collected at 200 g for 5 min by centrifugation at room temperature. The cells were resuspended in BPEL (supplemented with 8 mM CHIR99021 (S2924, Selleck Chemicals, Houston, TX, United States), 3.3 mM Y27632 (S1049, Selleck), and 1 mM β-mercaptoethanol (M3148,

TABLE 1 The primers used for RT-qPCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
GAPDH	GGCATGGACTGTGGTCATGAG	TGCACCACCAACTGCTTAGC
IL8	ACTGAGAGTGATTGAGAGTGGAC	AACCCTCTGCACCCAGTTTTC
KIM1	TGTCTGGACCAATGGAACCC	GGCAACAATATACGCCACTGT
MCP1	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT
IL1B	TTCGACACATGGGATAACGAGG	TTTTTGCTGTGAGTCCCGGAG
HIST1H3A	CTAGTGTTGGGTGTTCCGCT	CTGCCTTAGTGGCCAACTGT
SNORD3A	CGGTGACGGCTCTTGGGTTT	CGGGAAACGGCGACAAAA
miR-3615	CTCGGCTCCTCGCGGCTC	GCAGGGTCCGAGGTATTC
RPPH1	-GAGCTGAGTGCGTCCTGTC	TCAGGGAGAGCCCTGTTAGG
U6	ATTGGAACGATACAGAGAAGATT	GGAACGCTTCACGAATTTG

Sigma-Aldrich, St. Louis, MO, United States) and seeded into a 6-well ultra-low attachment plate (3,471, Corning). After 48 h, half of the medium was replaced with BPEL and CHIR99021 (8 mM). From day 3 onwards, embryoid bodies were resuspended in Stage II culture medium until day 14 and agitated daily to prevent excessive fusion. BPEL and Stage II medium (10–100–455, Aimingmed, Hangzhou, Zhejiang, China) were prepared as described by Przepiorski et al. (2018).

Cisplatin and celastrol treatment

Cisplatin (p4394, Sigma) and celastrol (C0869-10 MG, Sigma) were used for the nephrotoxicity testing. Cisplatin was reconstituted with a 0.9% sodium chloride solution to achieve a stock concentration of 0.5 mg/mL, and celastrol was dissolved in DMSO to reach a stock concentration of 10 mg/mL. The organoids were exposed to 50 μM cisplatin, as well as 100 nM, 200 nM, 500 nM, 1 μM, 2 μM, 5 μM, 10 μM, or 50 μM celastrol for a duration of 2 days starting from day 11 *in vitro*. In order to assess the kidney protective effect of celastrol, either 1 μM or 2 μM celastrol was co-administered with the cisplatin at a concentration of 50 μM. Organoids were harvested for immunostaining after fixation with 4% paraformaldehyde (P0099, Beyotime, Shanghai, China), or for qPCR and RNA-seq using TRIzol (15596018, Invitrogen, Carlsbad, CA, United States).

RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNAs was extracted using TRIzol, and 1 μg of total RNA was used for cDNA synthesis using the GoScript reverse transcription mix (A2790, Promega, Madison, WI, United States). Real-time PCR was performed using the SsoAdvanced SYBR Green Supermix (1725274, Bio-Rad, Hercules, CA, United States) in an real-time PCR machine (StepOnePlus, Thermo Fisher Scientific, Waltham, MA, United States). For the miRNAs, 2.5 μg of total RNA was reverse transcribed using All-in-One MiRNA Q-PCR Detection Kit (GeneCopoeia, Rockville, MD, United States). The primers used for the reaction are listed in Table 1.

RNA sequencing (RNA-seq) and bioinformatics analysis

To characterize the differential expression of RNA transcripts between the control, cisplatin-induced nephrotoxicity, and celastrol kidney protection groups, whole genome transcript sequencing was performed by (Aimingmed). Up-sequencing using the library construction method with rRNA removal allowed for simultaneous detection of mRNA expression levels. Gene expression was analyzed to assess the correlation between gene expression characteristics and differentially expressed genes within and between groups. Then, using pheatmap package and the hierarchical clustering was performed. The results were visualized using a heatmap. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment and Gene Ontology (GO) enrichment analyses were performed.

Immunofluorescence analysis

The organoids were washed with PBS and fixed with 4% paraformaldehyde (PFA) for 1 h at room temperature (RT). Afterwards, they were incubated in sucrose (30% w/v) in PBS overnight at 4°C. Then, the organoids were embedded in optimal cutting temperature (OCT) compound (4,583, Tissue-Tek, Torrance, CA, United States) and cryosectioned into 10-μm sections. The primary antibodies and dilutions used for immunofluorescence analysis are listed in Table 2. After washing thrice with PBS, the sections were incubated with the corresponding secondary antibodies (Table 2) and diluted in PBS for 1 h at RT. The nuclei were counterstained with DAPI for 10 min. Images were obtained using a confocal microscope (LSM710, Zeiss, Oberkochen, Germany).

Statistical analysis

We used the GraphPad Prism (Version 7; GraphPad Software La Jolla, CA, United States) to conduct statistical analysis. Data were

TABLE 2 The primary and secondary antibodies used for immunofluorescence analysis.

Antibody	Host species	Producer	Product code	Dilution
LTL	-	Vector Labs	FL-1321	1:300
KIM1	Goat	R&D Systems	AF1750	1:50
γH2AX	Rabbit	Cell Signaling	2577S	1:100
NPHS1	Sheep	R&D systems	AF4269-SP	1:200
CD31	Mouse	BD Biosciences	555444	1:200
SLC12A1	Rabbit	Sigma	HPA018107	1:200
PODXL	Mouse	R&D Systems	MAB1658	1:200
HNF1B	Rabbit	Sigma	HPA002083-100UL	1:200
CDH1	Mouse	BD Biosciences	610181	1:200
MEIS1/2/3	Mouse	Active Motif	39796	1:300
Donkey anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568	Donkey	Invitrogen	A10042	1:1000
Donkey anti-Sheep IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	Donkey	Invitrogen	A-11015	1:1000
Donkey anti- Goat IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647	Donkey	Invitrogen	A-21447	1:1000
Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568	Goat	Invitrogen	A-11004	1:1000

expressed as the mean ± SD. For comparison of more than three groups, Shapiro-Wilk test was used to check if a continuous variable follows a normal distribution. If $P > 0.05$, one-way ANOVA was applied; If $p < 0.05$, Kriskall-Wallis test was applied. Results were considered statistically significant with p values: $^{***}p < 0.001$, $^{**}p < 0.01$.

Flow cytometry

The organoids were washed with cold PBS and dissociated into single cells using Accumax (07921, STEMCELL). The cells were stained with propidium iodide (PI; Invitrogen) in PBS for 15 min at 4°C. The cells were washed with PBS and strained through a 100 μm mesh. Flow cytometry measurements were performed using a BD FACSCalesta cytometer (BD Diagnostics, Franklin Lakes, NJ, United States).

Transmission electron microscopy

The organoids were collected in a 2 mL tube and fixed with an electron microscopy fixation buffer consisting of glutaraldehyde (2.5%), paraformaldehyde (2%), and phosphate buffer (PB, 0.1 M, pH 7.4) overnight at 4°C. Post-fixation was incubated with 1% OsO4 in PB (0.1 M) for 1 h at 4°C. Kidney organoids were fixed in 1% Dehydrated in a graded series of ethanol solutions, and embedded in epoxy resin. Ultrathin sections (70 nm) were cut and stained with uranyl acetate and lead citrate. Slides were then imaged using an G2 Spirit transmission electron microscope (FEI Tecnai, Hillsboro, OR, United States).

Results

Generation and characterization of kidney organoids: morphological and molecular features

The process of kidney organoids self-organization is illustrated in Figure 1A. At 14 days of culture, the 3D structures presented tubular (red arrows) and glomerular (green arrows) formation (Figure 1B). These constructs expressed NPHS1, a critical protein primarily found in podocytes of the glomerulus, and CD31, a marker highly expressed in endothelial cells, indicating the development and/or maturation of the kidney vasculature (Figure 1C). Additionally, the organoids presented LTL (Green), a highly specific marker of the kidney's proximal tubules, and SLC12A1 (Red) staining, a marker for identifying the presence of thick ascending limb segments of the kidney (Figure 1D). Organoids contained multiple segmented nephron structures that resemble glomeruli and tubules marked by PODXL + podocytes (Figure 1E), HNF1B + tubules/collecting duct and CDH1+ distal tubules (Figure 1F), and MEIS1/2/3+ interstitial cells (Figure 1G). Typical images demonstrating the morphology of the kidney organoids exposed to celastrol or cisplatin under various conditions are shown in (Figure 1H). After the exposure of the kidney organoids to cisplatin, the organoids structure appeared partly collapsed. In addition, the collapsed structures were significantly reduced at celastrol was added.

We used 3D kidney organoid models to investigate the effects of cisplatin and celastrol on drug-induced kidney toxicity. The constructs were exposed to varying concentrations of the compounds to assess their effects on cell viability. Figure 2A

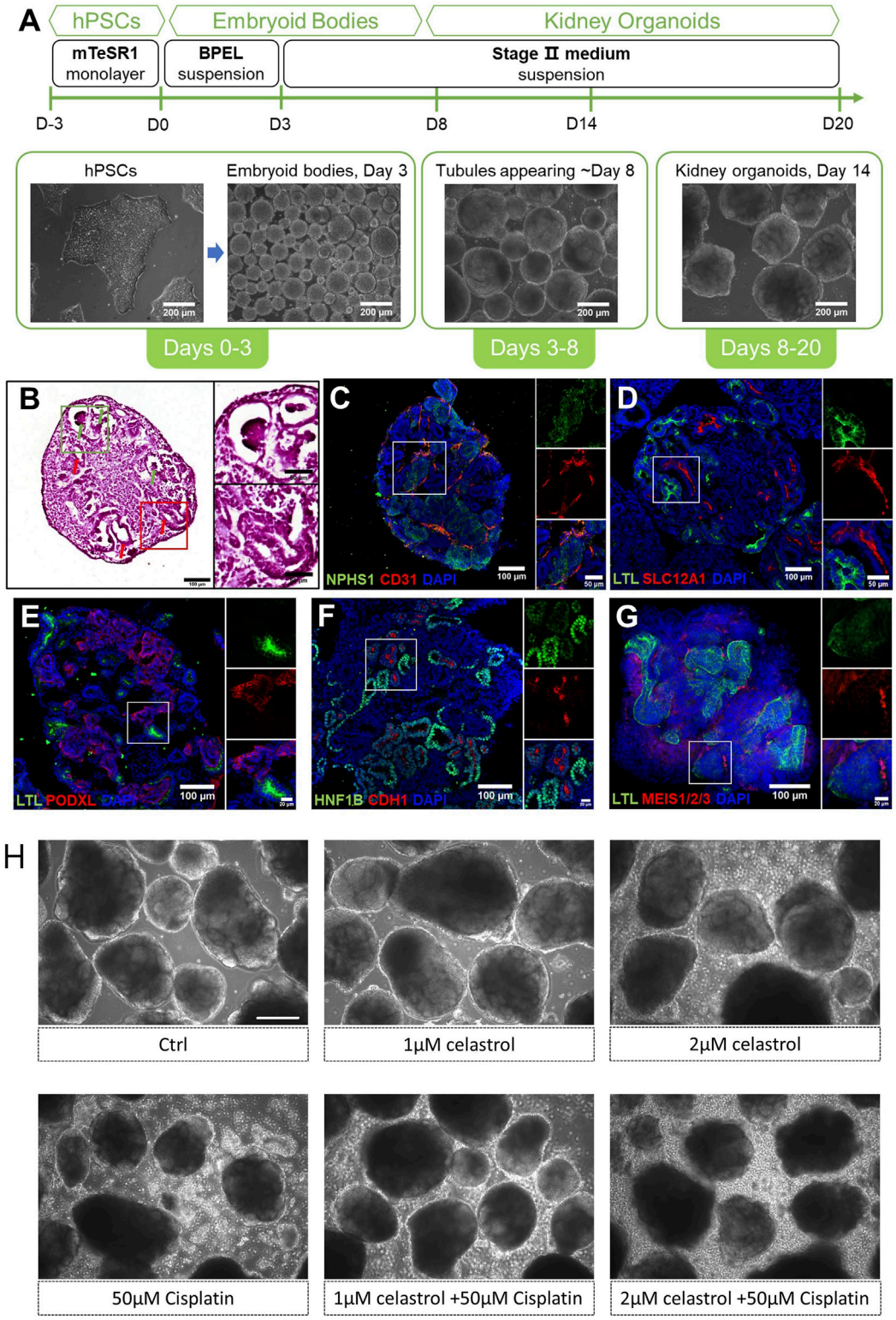

FIGURE 1
Self-organization and structural profiling of the kidney oragnoids. An overview of our differentiation method for kidney organoids. **(A)** An overview of our differentiation method for kidney organoids and representative images of kidney organoids at different stages. **(B)** Left: H&E staining of kidney organoid sections cultured to day 14, with the red arrows indicating tubular structures and green arrows indicating glomerular structures. Right: a zoom-in view of the red and green boxes. **(C–G)** Immunofluorescent staining of day 14 kidney organoids sections showing NPHS1+ podocytes and CD31^{+} endothelial cells, LTL + proximal tubules and SLC12A1+ thick ascending limb segments, PODXL + podocytes, HNF1B + tubules/collecting duct and CDH1+ distal tubules, and MEIS1/2/3+ interstitial cells. Corresponding zoom-in views of the white boxes are on the right of each image. **(H)** Representative bright field image (10X) of kidney organoids cultured with celastrol or cisplatin or co-treated with both compounds, Bar = 200 μm.

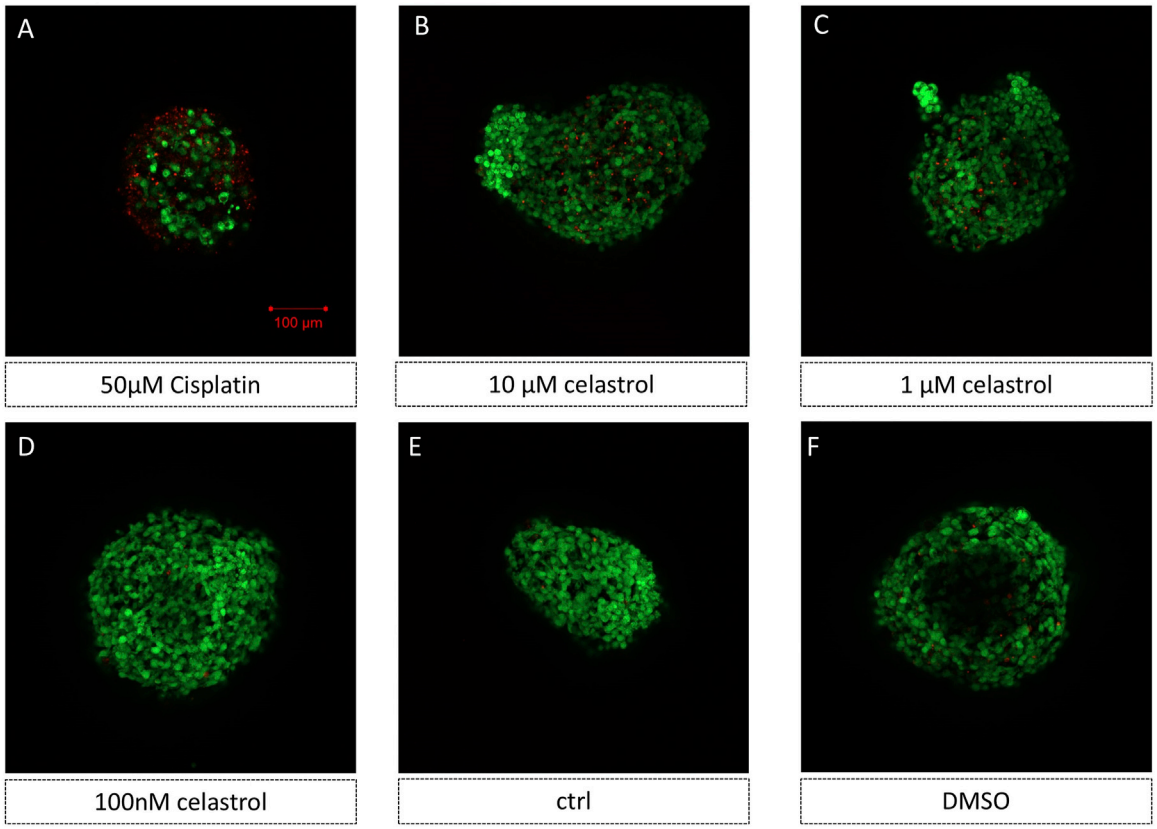

FIGURE 2
Representative images of 3D kidney oragnoids models showed cisplatin and celastrol induced kidney toxicity (AO/PI staining) n = 5. **(E)** kidney oragnoids not treated with drugs (control), **(A–D, F)** kidney oragnoids treated with: **(A)** 50 μM Cisplatin **(B)** 10 μM celastrol, **(C)** 1 μM celastrol, **(D)** 100 nM celastrol, and **(F)** DMSO.

shows a significant increase in cell death within organoids treated with 50 μM cisplatin, as evidenced by the increase in PI fluorescence, indicative of membrane damage and cell death. Moreover, the nephrotoxicity of celastrol at 10 μM (Figure 2B), 1 μM (Figure 2C) and 100 nM (Figure 2D) was significantly less pronounced than that observed in constructs treated with cisplatin-50 μM. Furthermore, the number of PI-stained cells in the 100 nM cL group (Figure 2D) was unnoticeable, similar to that found in Control (Figure 2E), and DMSO groups (Figure 2F).

Kidney organoid models reveal the dose-dependent response relationship of celastrol-induced citotoxicity

Kidney organoids were exposed to varying concentrations of celastrol (0.1, 0.2, 0.5, 1, 2, 5, and 10 μM) to assess dose-related toxic effects (Figure 3). The 3D constructs were stained with propidium iodide (PI) to determine cell viability. At 10 μM celastrol, the proportion of PI-positive cells reached 33.8%, compared to 7.7% in the control group, while the number of PI-stained cells in the groups treated with Celastrol >1uM remained comparable to that of the basal levels (Ctrl). The Flow cytometer assay findings demonstrated that celastrol induced cytotoxicity in a dose-dependent manner, as evidenced by the increased number of dead cells, with concentrations to produce low cytotoxicity and yet retain the beneficial effects at an optimal range of 1–2 μM.

The dose-dependent response of celastrol-induced cytotoxicity was compared with that of cisplatin treatment. Flow cytometry analysis revealed that cisplatin at 50 μM resulted in only 19.7% of cells maintaining a normal structure, in stark contrast to the 55.8% observed in the control (Ctrl) and DMSO groups. Interestingly, Celastrol at 0.2 μM and 1 μM concentrations demonstrated normal structure cell percentages comparable to those in the DMSO and control groups (56.3%). However, at higher concentrations of 5, 10, and 50 μM, celastrol induced a dose-dependent decrease in the percentage of cells with normal structure, underscoring its nephrotoxicity at elevated doses (Figure 4).

To assess the impact of renal injury induced by individual treatments with cisplatin or celastrol, as well as their combined application, we adopted a holistic measurement strategy that encompasses the simultaneous quantification of several key renal mRNA markers. Specifically, we have focused on the mRNA levels of Interleukin-8 (IL-8), Interleukin-1 beta (IL-1β), Kidney Injury Molecule-1 (KIM-1), and Monocyte Chemoattractant Protein-1 (MCP-1), as these biomarkers are indicative of renal stress, inflammation, and injury (Figure 5). IL-1β and IL-8 are central to the kidney's inflammatory response, with elevated mRNA levels indicating activation of inflammatory pathways typical of nephrotoxic agents, such as cisplatin. Remarkably, the mRNA

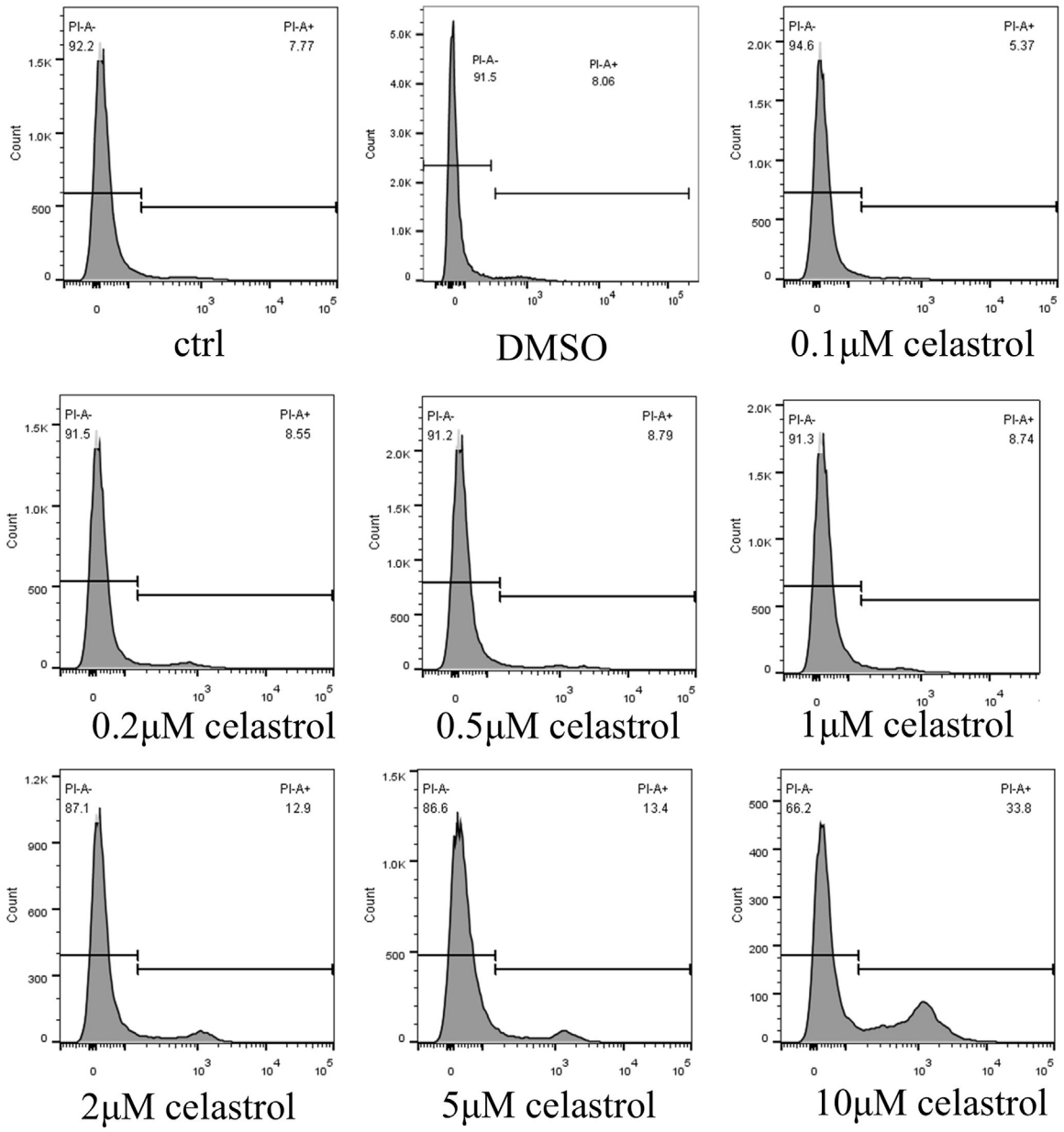

FIGURE 3
Dose-dependent Kidney Toxicity Induced by Celastrol evaluated by Flow cytometer. The rate of death cell after administration of DMSO or celastrol at 0.1, 0.2, 0.5, 1, 2, 5, or 10 μM for 2 days in kidney organoids. The dose-dependent response of celastrol-induced cytotoxicity was evaluated.

levels of IL-1β, and IL-8 in the groups treated with a combination of 50 μM cisplatin and 1 (1+cp), and particularly 2 (2+cp) μM celastrol, were similar to those observed in the control groups (Ctrl, DMSO). Moreover, the mRNA expression levels in the 1+cp and 2+cp groups were significantly lower than those seen in the 10uM Celastrol or Cisplatin 50uM the transcriptional expression of KIM-1, a specific marker for kidney proximal tubule injury, showed similar expression patterns and were downregulated to basal levels when organoids were subjected to 50 μM cisplatin combined with 1 and 2 μM celastrol. However, MCP-1, known to recruit monocytes and macrophages to inflammation sites that can exacerbate tissue damage, also presented similar expression patterns to those observed in the previously described biomarkers. However, 1+cp did not bring MCP-1 mRNA levels to control levels. Taken together, by measuring these biomarkers, we can gain insights into the mechanisms of celastrol-induced nephrotoxicity, predict the extent of kidney damage, and potentially guide dosage adjustments to mitigate adverse effects, thus providing a holistic view of the nephrotoxic potential of the compound.

Celastrol alleviated renal glomerulus and proximal tubules injury

Figure 6A shows representative images of kidney injury markers (KIM-1 is a specific and sensitive biomarker of proximal tubules of

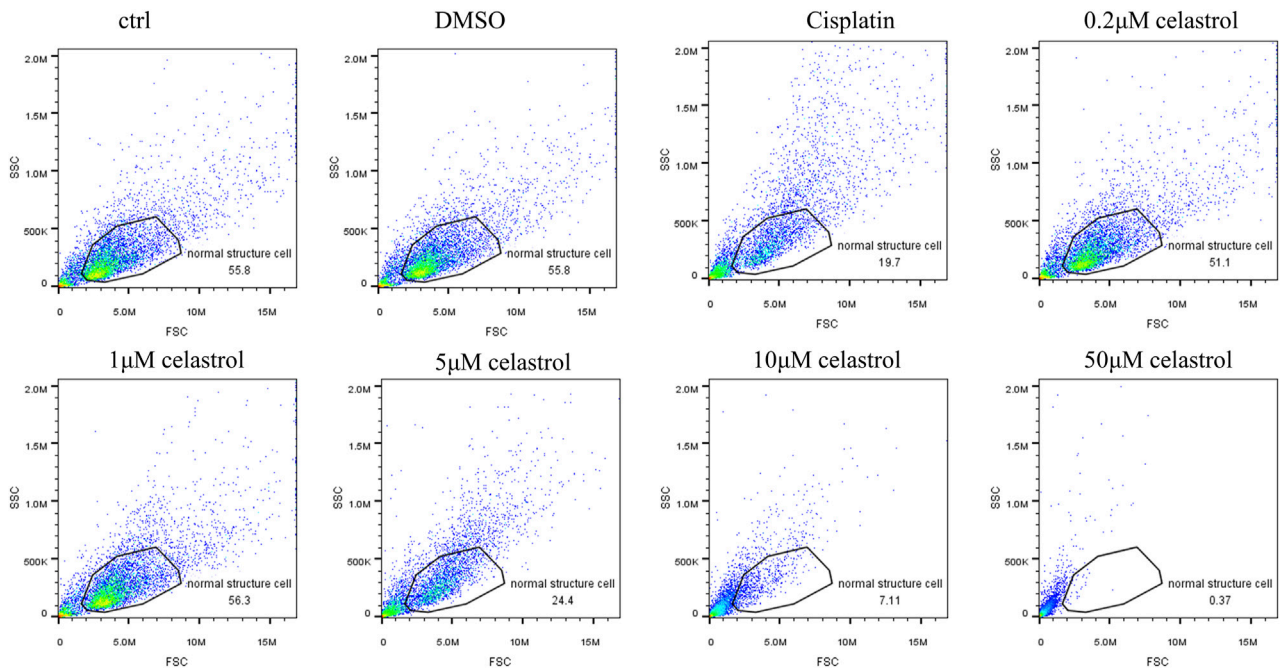

FIGURE 4
Flow cytometry analysis rate of normal cell structure after administration of celastrol at 0.2, 1, 5, 10μM, or 50 mM for 2 days in kidney organoids. The dose-dependent response of celastrol-induced cytotoxicity was compared with that of cisplatin treatment.

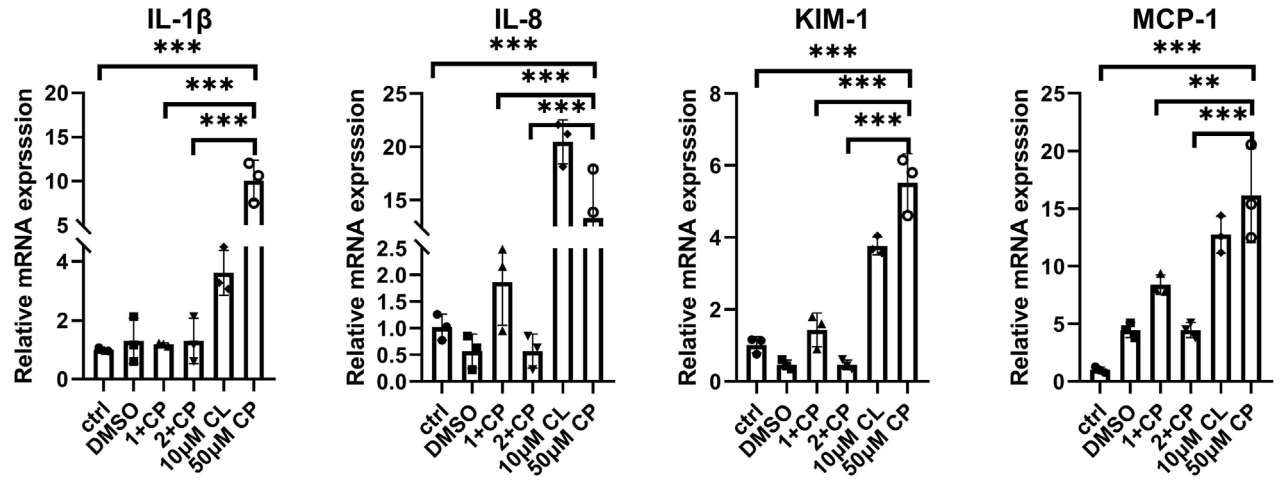

FIGURE 5
Expression of kidney inflammatory and injury mRNA biomarkers in kidney oragnoids incubated with 1 μM celastrol+50 μM cisplatin, 2 μM celastrol+50 μM cisplatin, 10 μM celastrol, and 50 μM cisplatin for 2 days n = 3. ctrl, control; 1+cp, 1 μM celastrol+50 μM Cisplatin; 2+cp, 2 μM celastrol +50 μM Cisplatin; 10μM CL, 10 μM celastrol; cp, Cisplatin. (n = 3/group, error bars represent the SD, **P ≤ 0.01; ***p ≤ 0.001 in the different groups compared with the cp group using a One-way ANOVA test).

kidney injury, γH2AX indicates DNA damage) in LTL-stained kidney organoids following the administration of celastrol at concentrations of 1 μM and 2μM, cisplatin at 50μM, or a combination of both drugs, for a duration of 2 days. The fluorescence intensity was showed in (Figure 6B), determined using the ImageJ software, revealed the cytotoxic effects of cisplatin at 50μM, as indicated by the elevated expression of both renal damage markers (Supplementary Figure 1). Notably, the co-administration of cisplatin 50 μM with 1μM and 2 μM celastrol not only mitigated drug-induced kidney injury, but also demonstrated protective actions. These findings suggest that 1 and 2 μM celastrol could potentially offer a novel therapeutic strategy that balances the potent anticancer action of cisplatin with the cytoprotective benefits of celastrol, thereby enhancing the overall safety and efficacy of cancer treatment.

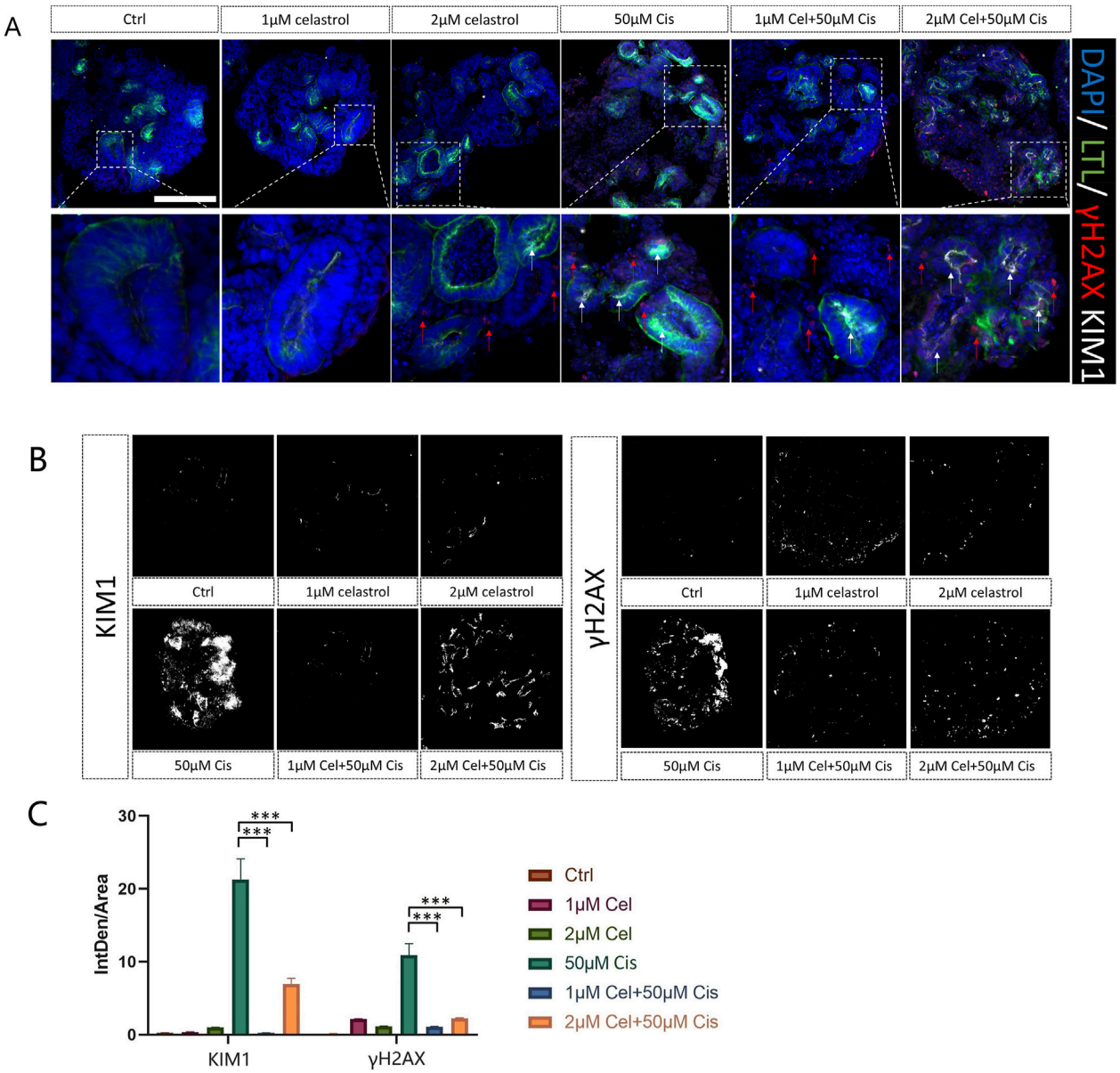

FIGURE 6
(A) Representative images of kidney injury (γH2AX, KIM1) in LTL of kidney organoids after administration of celastrol at 1 and 2 μM, or Cisplatin 50 μM for 2 days n = 3. Bar = 100 μm. **(B)** Quantification of γH2AX and KIM1 fluorescence intensity. n = 5. **(C)** The fluorescence intensity vs. area. Ctrl, control; 1 μM Cel, 1 μM Celastrol; 2 μM Cel, 2 μM Celastrol; 50 μM Ci, Cisplatin; 1 μM Cel+50μMCis,1 μM celastrol+50 μM Cisplatin; 2 μM Cel+50μMCis, 2 μM celastrol +50 μM Cisplatin; (n = 5/group, error bars represent the SD, ***p ≤ 0.001 in the different groups compared with the 50 μM Cisplatin group using a Kriskall-Wallis test).

In this study, human pluripotent stem cells derived kidney organoids comprised of not only complex interacting component cell types, but also some distinct segmenting nephrons, including distal tubule, proximal tubule, foot processes and podocytes of the glomerulus (Figure 1). With such various of kidney cell types, the advantages for use of these nephrons for nephrotoxicity screening have significantly promoted (Figure 7). Cisplatin induced-kidney injury was characterized by TEM, in cisplatin group, clear damages in tubular cells were identified, such as loss of cells shape, necrosis of renal tubular epithelium and vacuoles formation. 1 μM celastrol + cisplatin group resulted the significant injury recovery in tubular cells comparing with others.

Downregulation of SNORD3A and mir3615 by celastrol mitigates cisplatin-induced nephrotoxicity in kidney organoids

For the RNA-seq result, compared 2 μM celastrol + cisplatin vs. Cisplatin group, GO analysis shown the most differentially expressed functional pathways are the renal system, renal tubular secretion and excretion function based on the GO database, indicating celastrol regulate renal tubular secretion and excretion function with protective effect on glomerulus. Meanwhile, Figure 8 shows the significant upregulation of SNORD3A in kidney organoids treated with cisplatin (Cis), with high expression levels

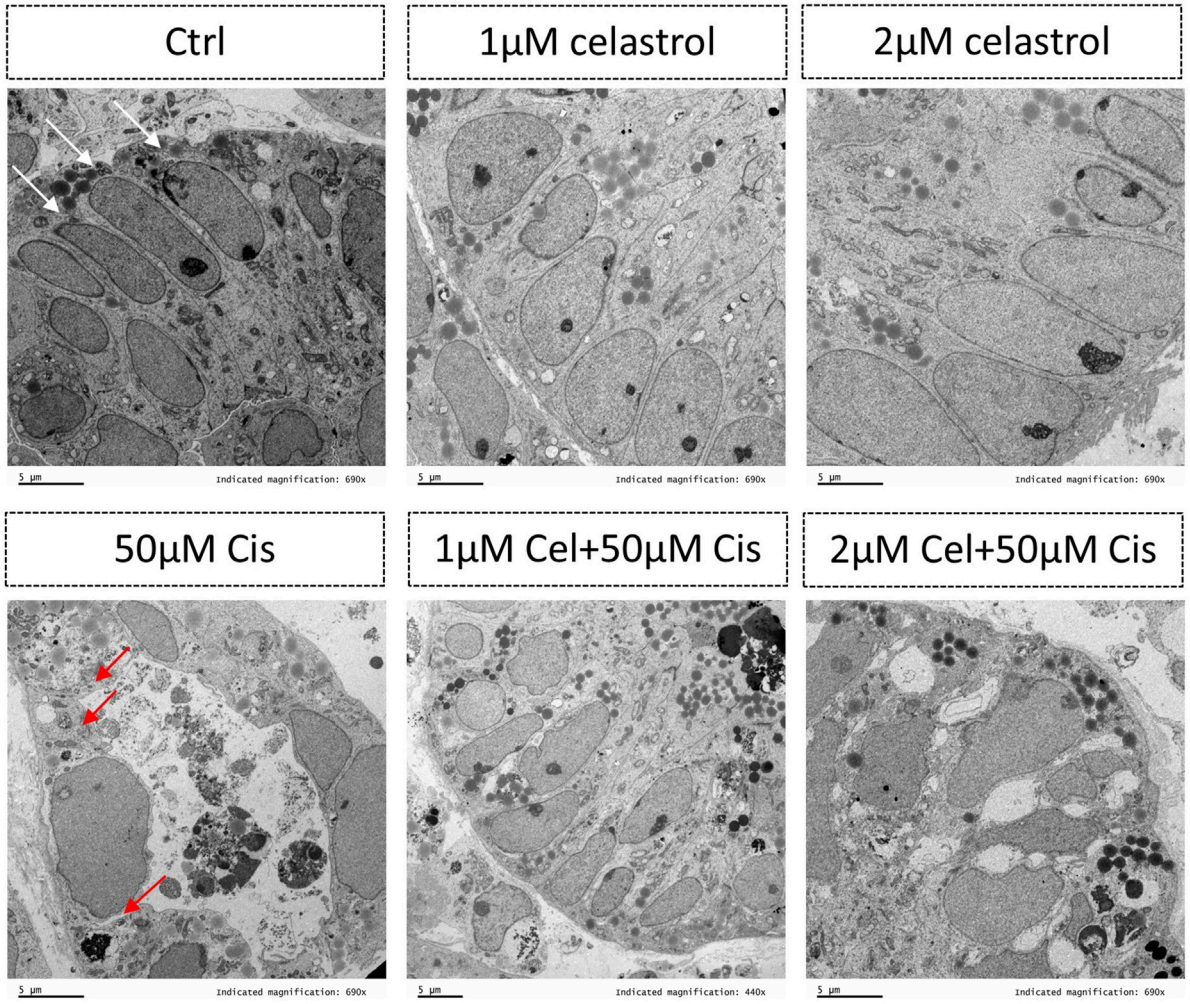

FIGURE 7
Transmission electron microscopy showing the presence of tubular cells within the kidney organoids. The white arrows indicate normal tubular cells while red arrows indicating damaged tubular cells after injury. Ctrl, control; Cel, celastrol; Cis, cisplatin.

correlating with nephrotoxicity. SNORD3A was primarily enriched in tubular epithelial cells in response to acute kidney injury (AKI) in tubular epithelial cells. We also observed a dramatic decline in SNORD3A expression in the celastrol + cisplatin group (2Cel-Cis) (Figure 8C), indicating that celastrol played a pivotal role in the inhibition of cisplatin-induced nephrotoxicity. Previous studies have demonstrated that miRNAs are involved in the pathophysiology of AKI, and aberrant miRNA expression levels serve as biomarkers for diagnosing AKI miRNA signatures. Our RNA-seq and RT-qPCR results showed that miR-3615 was positively associated with cisplatin-induced kidney injury. Moreover, miR-3615 was significantly downregulated in the 2Cel-Cis, further indicating its positive relationship with cisplatin-induced nephrotoxicity (Figure 8C). Furthermore, RPPH1, which is upregulated in diabetic nephropathy via an interaction with Gal-3, and the HIST1H3A gene, both presented expression patterns consistent with what we've observed for the previous analyzed genes, also indicating a possible relationship with the protective actions of Cis/Cel cotreatment (Figure 8C).

Discussion

Here, we present a comprehensive evaluation of kidney organoids self-organization using our refined differentiation method. This evaluation shows their structural and molecular characteristics, which closely mimic those of the native kidney tissues. The constructs displayed markers indicative of renal development, such as NPHS1+ podocytes and $CD31^{+}$ endothelial cells, which are crucial for glomerular and vascular formation (Figure 1D). Additionally, SLC12A1+ and LTL + staining confirmed the differentiation of thick ascending limb segments and proximal tubules (Figure 1E), which are essential components of the renal filtration system. At the same time, there are a variety of techniques that were utilized to the measurement of function and structure of kidney organoids, including immunofluorescence, RNA-seq, Flow cytometer, and TEM.

The kidney organoid model was successfully utilized to assess the nephrotoxic effects of cisplatin and cisplatin-induced

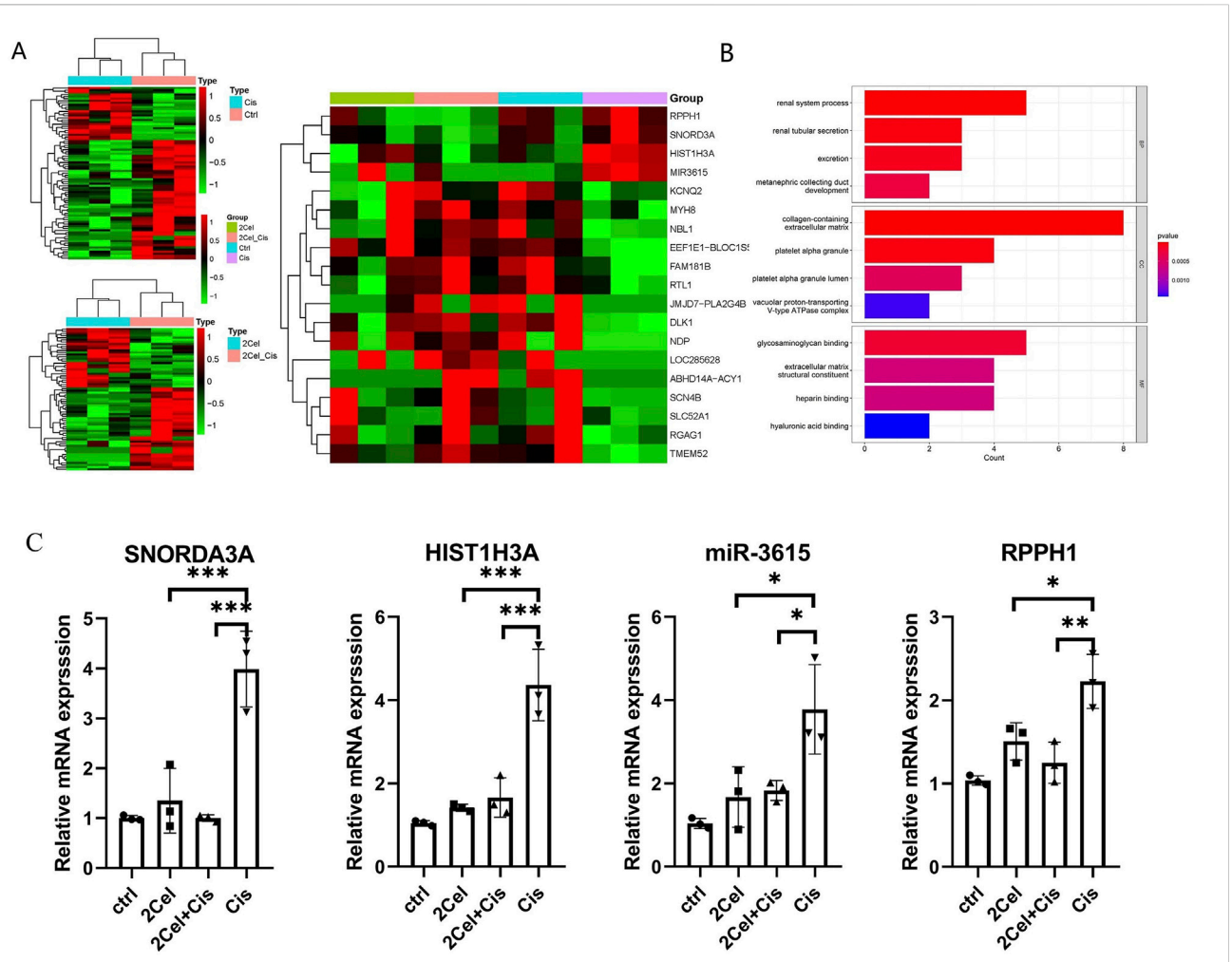

FIGURE 8
(A) Heatmap of up-and downregulated DEGs of different groups (Cisplatin vs. Control, 2 μM Cel + Cis vs. Cis, Four groups compare). **(B)** The GO barplot of 2 μM celastrol + Cisplatin vs. Cisplatin group. The diagrams showing the most differentially expressed functional pathways regarding the renal system, renal tubular secretion and excretion based on the GO database. **(C)** Validation of mRNA and miRNA by RT-qPCR. Relative expression level of HIST1H3A and specific ncRNAs SNORD3A, miR-3615, and RPPH1 in kidney organoids treated with 2 μM celastrol, 50 μM Cisplatin or 2 μM celastrol+50 μM Cisplatin for 2 days. Relative quantifcation was determined by normalization to GAPDH or U6. (n = 3/group, error bars represent the SD, *P ≤ 0.05; **P ≤ 0.01; ***p ≤ 0.001 in the different groups compared with the cp group using a One-way ANOVA test).

nephrotoxicity reversal by cotreatment with celastrol. Consistent with the literature (Tang et al., 2023; Belmonte-Fernández et al., 2023), in our study, we observed a significant increase in cell death in organoids treated with 50 μM cisplatin (Figure 2A) compared to those in the celastrol-treated and control (ctrl, DMSO) groups. Celastrol's dose-dependent nephrotoxicity (Figure 3) is a novel observation that adds to the existing body of knowledge on the compound's biological effects. Lower concentrations of celastrol (1–2 μM) showed minimal toxicity, suggesting a potential therapeutic window where celastrol could exert its anti-inflammatory and anti-neoplastic effects without causing significant kidney injury. The comparative analysis of cytotoxic effects between cisplatin and celastrol treatments (Figure 4) revealed that celastrol, at certain concentrations, could mitigate cisplatin-induced nephrotoxicity. This is a significant finding, as it suggests that celastrol may have a protective role against kidney injury, possibly through its antioxidant and anti-inflammatory properties (Allison et al., 2001; Yang et al., 2006). Though, potential toxicity restricts its further application. Immortalized cell models like human proximal tubule epithelial cell line (HK-2) and mouse renal tubule epithelial cells (RTECs) are commonly used for nephrotoxicity assessment. However, absence of essential molecular structures and cellular components makes its data not easily translated to equivalent values *in vivo*. Significant differences in celastrol safety values were found between cell lines and kidney organoids. It was reported that the highest tolerant values of celastrol on HK-2 and RTECs were 50 nM ((Yu et al., 2018)). The dose we used in kidney organoids in the present study was much more than above values. The celastrol concentrations to generate low nephrotoxicity and yet remain the beneficial effects at an range from 1 to 2 μM.

The combination of cisplatin and celastrol resulted in downregulation of inflammatory and injury biomarkers (Figure 5), indicating a potential synergistic effect. Normalization of IL-1β, IL-8, KIM-1, and MCP-1 mRNA levels in co-treated organoids suggests that celastrol may modulate the inflammatory

response and promote tissue repair, a promising avenue for future research. Moreover, fluorescence intensity analysis of γH2AX and KIM-1 has shed light on the potential benefits of co-administering cisplatin (50 μM) with celastrol (1–2 μM) in mitigating drug-induced kidney injury. This co-administration may contribute to the complex mechanisms by which celastrol provides cytoprotection. Phosphorylation of the Ser-139 residue on the histone variant H2AX, resulting in the formation of γH2AX, represents an early cellular response to DNA double-strand breaks. Detection of this phosphorylation event is recognized as a highly specific and sensitive molecular marker for monitoring the onset and resolution of DNA damage. Importantly, this marker has been linked to oxidative stress and the production of reactive oxygen species (ROS) (Mah et al., 2010). Therefore, upregulation of γH2AX in response to cisplatin may serve as an indicator of oxidative stress and ROS production during drug-induced kidney injury (DIKI).

Additionally, we examined the expression of several transcription factors. Among them, HIST1H3A and the non-coding RNAs (ncRNAs) SNORDA3A, miR-3615, and RPPH1 showed similar expression patterns, which were positively correlated with the cytotoxicity features of 50 μM cisplatin and the protective action of 2 μM celastrol. HIST1H3A is one of the genes that encode the histone H3.1 protein, and histone modifications have been implicated in both the development and progression of kidney diseases as well as AKI (Kato and Natarajan, 2019; Wang et al., 2022; Pan et al., 2024). For instance, phosphorylation of histone H3 on serine residue 10 (H3Ser10) has been linked to endothelial activation in diabetic kidney disease, facilitating the recruitment of inflammatory cells that contribute to kidney injury and fibrosis (Alghamdi et al., 2018). On the other hand, Zhu et al. (2024) observed that deficiencies in SNORDA3A exhibit a mitigating effect on the stimulator of interferon gene (STING)-associated ferroptosis phenotypes and the progression of kidney tubular injury. Mechanistically, SNORDA3A regulates the STING signaling axis by promoting STING gene transcription, and the administration of SNORDA3A antisense oligonucleotides represents a significant therapeutic advantage in a mouse model of AKI (Zhu et al., 2024). Consistent with Zhu's study, we observed a significant upregulation of SNORD3A in response to cisplatin-induced nephrotoxicity and its subsequent downregulation upon celastrol cotreatment, suggesting that the protective effects of celastrol at 1 or 2 μM might be partially related to the inhibition of the progression of tubular injury (Figure 8). Although the link between miR-3615 and kidney injury has not been well documented in the available scientific literature, we analyzed its transcriptional expression. These results indicated that miR-3615 was positively associated with cisplatin-induced kidney injury and celastrol-induced cytoprotective action. Furthermore, RPPH1 (ribonuclease P RNA component H1), a critical component of the ribonuclease P complex involved in the maturation of tRNA molecules by cleaving their 5′leader sequences, showed expression patterns similar to those of the other ncRNAs. RPPH1 plays a fundamental role in cellular RNA processing and is essential for proper functioning of the ribonuclease P enzyme complex. However, in the context of AKI and DIKI, particularly cisplatin-induced nephrotoxicity, the specific mechanisms by which RPPH1 might be involved have not yet been elucidated.

Taken together, the potential therapeutic advantages of targeting HIST1H3A and specific ncRNAs such as SNORD3A, miR-3615, and RPPH1 further support the complex interplay between these genomic elements and drug-induced nephrotoxicity. With the rise of organoid technologies, it has become possible to studying various diseases that affect the kidneys as well as a preclinical model for drug toxicity screening and to investigate the structure and molecular changes occurring in a more physiologically relevant environment.

Conclusion

Our kidney organoid model has been proven to be an invaluable tool for advancing our understanding of nephrotoxicity. Its robustness lies in its ability to closely mimic the structural and functional characteristics of native kidney tissues, providing a reliable and controllable environment for studying the mechanisms of DIKI, such as those induced by cisplatin.

Moreover, our findings underscore the significance of a cotreatment approach using cisplatin and celastrol. at concentrations of 1 and 2 μM, celastrol has been demonstrated to complement the full anti-neoplastic potential of cisplatin in cancer treatment with its own cytoprotective actions. This synergistic combination offers a promising avenue for enhancing the therapeutic efficacy of cisplatin, which is a widely used chemotherapeutic drug. By reducing its cytotoxic side effects on the kidneys while maintaining its anticancer potency, it is possible to envision a new paradigm in cancer therapy that is both effective against tumours and protects normal tissues. However, the therapeutic window of celastrol is very narrow (from 1 to 2 μM), associated with the occurrence of side effects. Further research is warranted to fully realize the clinical potential of these findings and studies on human clinical trial are required.

Data availability statement

The raw data presented in the study are deposited in the National Genomics Data Center, accession number OMIX007089; available at https://ngdc.cncb.ac.cn/omix/releaseList.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

CS: Conceptualization, Funding acquisition, Writing–original draft. QW: Data curation, Methodology, Resources, Visualization, Writing–original draft. XY: Data curation, Investigation, Validation, Writing–original draft. YZ: Project administration, Writing–review and editing. HX: Project administration, Supervision, Writing–review and editing. CP: Methodology, Visualization, Writing–review and editing. HL: Methodology, Visualization, Writing–review and editing. CW: Investigation, Project

administration, Supervision, Writing–review and editing. MY: Investigation, Resources, Writing–review and editing.

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Conflict of interest

Authors YZ, HX, and MY were employed by Hangzhou Aimingmed Organoids Bank.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2025.1464525/full#supplementary-material

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Patient-derived organoids reveal marked heterogeneity in chemosensitivity profiles of colorectal cancer and a potential association with HER2 status

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This study aimed to evaluate the sensitivity and heterogeneity of standard first-line chemotherapy regimens for colorectal cancer (CRC) using patient-derived tumor organoids (PDTOs). Drug sensitivity testing in 15 CRC PDTOs revealed varying proportions of samples classified as sensitive (inhibition rate >50%) across different regimens: FOLFIRI (60%), FOLFOX (40%), CAPEOX (26.7%), and 5-Fluorouracil (26.7%). Furthermore, exploratory analysis indicated that for FOLFIRI and FOLFOX regimens, HER2(1+) status was significantly associated with lower inhibition grades compared to HER2(0) status ($P < 0.05$), implying a potential impact on the level of drug response. These findings demonstrate significant heterogeneity in the response of CRC PDTOs to first-line chemotherapies. Furthermore, at the organoid level, a low HER2 expression status may be associated with the heterogeneity of responses observed with specific drug regimens.

KEYWORDS

colorectal cancer, patient derived tumor organoids, chemotherapy, drug sensitivity, HER2 heterogeneity

Introduction

Clinical responses to standard chemotherapy regimens for CRC exhibit significant inter-patient variability (Fyfe, 2023; Picco et al., 2021), highlighting the lack of reliable predictive biomarkers to guide personalized therapy. PDTOs have emerged as advanced preclinical models capable of preserving key histopathological and genetic features of the original tumor (Booij et al., 2022; Veninga and Voest, 2021), thereby providing a valuable platform for assessing individual drug sensitivities. This study employed PDTOs established from 15 distinct CRC patients to meticulously profile their response landscape to a panel of standard-of-care chemotherapeutic drugs and combinations, illustrating the substantial inter-patient heterogeneity in chemosensitivity and the considerable potential of PDTOs as preclinical models for drug efficacy screening.

Human epidermal growth factor receptor 2 (HER2) is widely recognized as an effective target for targeted therapy in colorectal cancer (Suwaidan et al., 2022); however, its role in the context of conventional chemotherapy has received limited investigation. To date, no definitive reports have demonstrated that HER2 status significantly modulates

chemotherapeutic outcomes at the population level. Nevertheless, at the cellular level, studies have indicated a discernible association between HER2 positivity (defined as immunohistochemistry [IHC] 3+, or IHC 2+ coupled with fluorescence *in situ* hybridization [FISH] amplification) and resistance to chemotherapeutic agents such as 5-fluorouracil (5-FU) (Long et al., 2022) and oxaliplatin (Pirpour Tazehkand et al., 2018). This suggests that the relationship between HER2 expression status and chemosensitivity in CRC is complex and context-dependent. To further elucidate the association between HER2 status and sensitivity to commonly used chemotherapeutic agents in CRC, we conducted an exploratory analysis using PDTOs, a model system whose biological characteristics are considered intermediate between *in vivo* patient tumors and conventional *in vitro* cell lines. This analysis examined potential correlations between observed drug sensitivity patterns and HER2 expression status (0 vs 1+), aiming to explore the multifaceted relationship between HER2 status and drug sensitivity.

Materials and methods

Colonic tumor organoids culture

Tumor samples for PDTOs culture were obtained from surgical resections of primary stage II or III CRC. Following acquisition, tumor tissue was isolated and digested using a digestive solution (MasterAim®). 50,000–60000 isolated cells mixed with cold Matrigel Basement Membrane Matrix (CORNING) and 50 μL drops of Matrigel-cell suspension were allowed to solidify on prewarmed 24-well suspension culture plates at 37°C for 30 min. Upon complete gelation, 500 μL of organoid medium was added to each well and plates were transferred to humidified 37°C/5% CO_2 incubator. The pellet was resus pended with colonic tumor organoid culture medium (MasterAim®Colorectal Cancer Organoid Kit, 10–100–066). The culture was replenished with fresh media every 3–4 days during organoid growth. Dense cultures with organoids were usually passaged with a split ratio of 1:3 every 2–3 weeks by dissociation with TrypLE Express (Gibco) and re-seeded into new Matrigel.

Organoid drug screening

10 μL of Matrigel was dispensed into 384-wellmicroplates and allowed to polymerize. Cells from organoid were plated (1.5–2 × 10^3 per well) and cultured in 384-well culture plates (CORNING) for 48 h, and drugs were added to the culture medium at a final concentration. Test agents were primarily selected based on first-line chemotherapy regimens recommended by the Chinese Society of Clinical Oncology guidelines for CRC (2024), including: CAPEOX (Capecitabine + Oxaliplatin), FOLFOX (Calcium Folinate + 5-Fluorouracil + Oxaliplatin), FOLFIRI (Calcium Folinate + 5-Fluorouracil + Irinotecan), FOLFOX + Cetuximab, 5-Fluorouracil, and Cetuximab. The concentration for each individual drug component was: Oxaliplatin 1μM, 5-Fluorouracil 5μM, Irinotecan 5μM, Calcium Folinate 5μM, Capecitabine 5μM, and Cetuximab 1 μM. After 3 days of drugs incubation, cell viability was assayed using Cell Titer-Glo 3D Reagent (Promega) in accordance with the manufacturer's instructions. 0.1% dimethyl sulfoxide was used as a control. When the ratio of the average level of cell viability in the presence of the drugs (n = 2) compared to the control (n = 2) was under 0.7, and the suppressive effect was considered to be significant. Inhibition rates were categorized into four grades for analysis: Grade A (≥70%), Grade B (50% to <70%), Grade C (30% to <50%), and Grade D (<30%). Drug inhibition rates were calculated using the formula: Inhibition Rate (%) = [1 - (Chemiluminescence_drug - Chemiluminescence_blank)/(Chemiluminescence_negative_control - Chemiluminescence_blank)] * 100%. Compared to the drug group, the negative control group received no drug, and the blank group contained no organoids; all other conditions were kept the same.

Histological analysis and immunohistochemistry

Tumor tissue and organoids were fixed with 4% paraformaldehyde overnight, washed, and embedded into paraffin blocks. Sections (four to five μm) were deparaffinized andstained with hematoxylin and eosin (H&E) for histological analysis. For Immunohistochemistry, after sections were made and hydrated, they were incubated with blocking buffer with H_2O_2 for 15 min and boiled with citrate (pH = 6.0). After cooling down, sections were treated with pre-blocking buffer and incubated with primary antibodies at 4°C overnight. Sections were incubated with secondary antibodies and HRP (horse radish peroxidase). Primary antibodies were used including CK7 (HUA Bio, ET1609-62), CK20 (HUA Bio, ET1601-8) and Epcam (CST, 2929 S).

Descriptive statistics were employed to summarize sample characteristics, clinical information, and inhibition rate results across different drug regimens. Associations between inhibition rate grades and HER2 status derived from immunohistochemistry were evaluated using the Mann-Whitney U test.

Results

A total of 16 CRC tumor tissue samples were obtained, from which 15 PDTOs were successfully established. Subsequent immunohistochemistry analysis confirmed good concordance between these organoids and their corresponding parental tissues (Figure 1). The cohort comprised six male and nine female patients, with an age range of 51–80 years. Tumor staging revealed 7 cases of stage II and 8 cases of stage III adenocarcinoma. Immunohistochemistry indicated proficient mismatch repair (pMMR) status in all samples, with expression of MLH1 (+), MSH2(+), MSH6(+), and PMS2(+). HER2 status was negative (score 0) in 10 cases and weakly positive (score 1+) in 5 cases.

The organoids exhibited significant morphological changes following drug treatment (Figure 2). Significant heterogeneity was observed in the inhibition rates of the different PDTOs in response to the tested drugs (Figure 3; Table 1). The drug regimen with the highest mean inhibition rate was FOLFIRI (mean ≈51%, SD ≈ 23%), followed by FOLFOX (mean ≈41%, SD ≈ 21%). The lowest mean inhibition was observed with Cetuximab monotherapy (mean ≈20%, SD ≈ 17%). For the majority of patients, the inhibition rates of combination therapy were higher than those of monotherapy. However, in one sample, the inhibition rate for 5-Fluorouracil

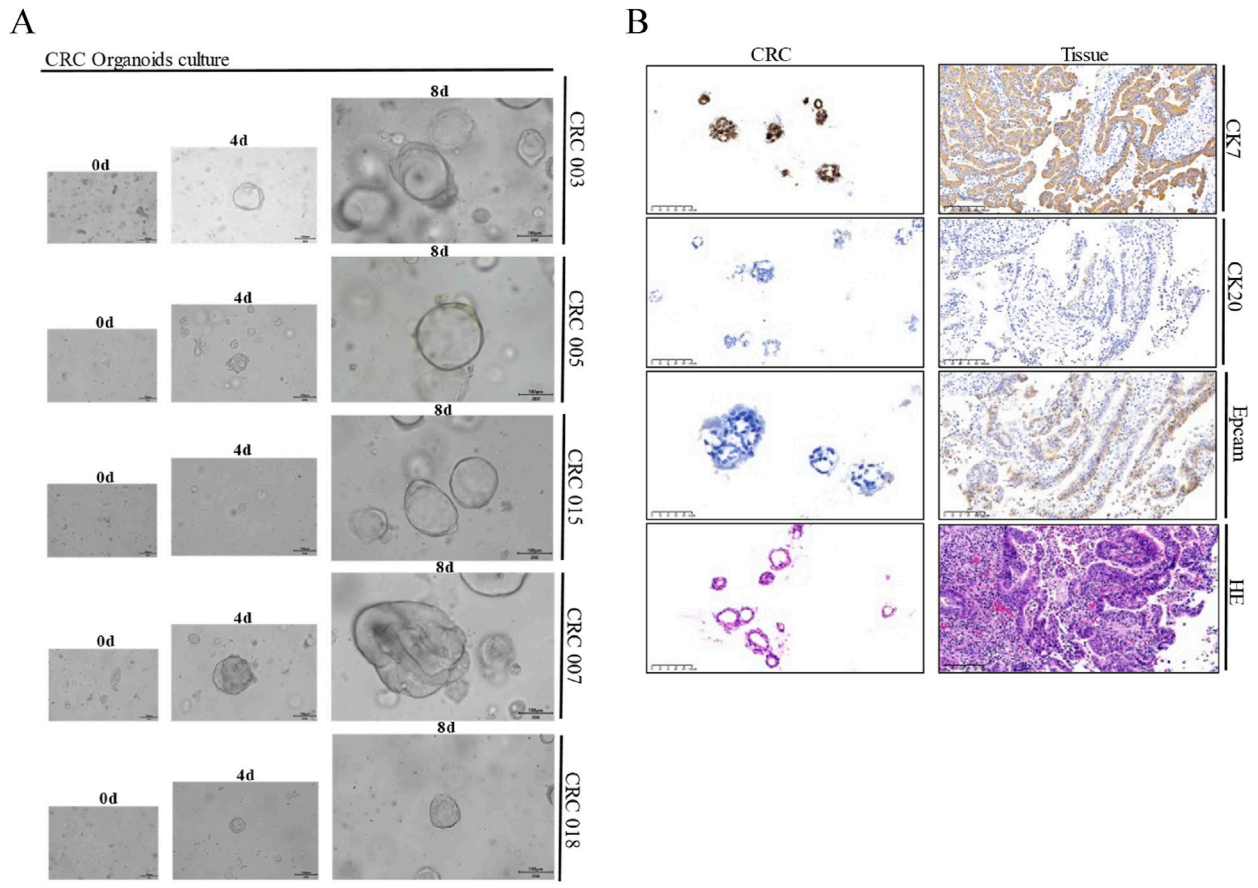

FIGURE 1
Representative morphology and comparative immunohistochemistry of PDTOs and corresponding parental tissues. **(A)** Representative bright field images of CRC tumor organoids from seven different patients. CRC organoids tends to more shaped thin-walled cystic structures. Case ID was named according to experimental specimen number. Scale bar: 100 μm; **(B)** Representative H&E and immunohistochemistry staining of intestine tumor and derived organoid lines. Tissues generally present tumor epithelium surrounded by mes enchymal and inflammatory cells, while organoids are exclusively epithelial with tumor cell organization being remarkably well conserved. Scale bar:100 μm.

monotherapy was substantially higher than that observed with combination therapies.

Stratified analysis revealed that for the FOLFOX, FOLFIRI, and FOLFOX + Cetuximab combination regimens, HER2(1+) status was significantly associated with lower inhibition grades (indicating poorer drug efficacy) compared to HER2(0) status ($P < 0.05$). Conversely, for CAPEOX, single-agent 5-Fluorouracil, and single-agent Cetuximab, although a trend towards poorer efficacy in the HER2(1+) group was observed, this difference did not reach statistical significance within our study's sample size ($P > 0.05$). Detailed data are presented in Table 2.

Discussion

This study successfully established PDTOs from stage II and III colorectal cancer patients, demonstrating good concordance with parental tissues. These PDTO models clearly illustrated significant heterogeneity in sensitivity among CRC patients to standard first-line chemotherapy, supporting the potential of PDTOs as a preclinical platform for capturing individual variations and exploring personalized therapeutic strategies. Notably, although FOLFIRI exhibited the highest *in vitro* response rate (60% Grade A + B) in our PDTOs, this contrasts with current guidelines generally not recommending its use in the adjuvant setting for stage II/III CRC (Harada and Sakamoto, 2022), a discrepancy also noted in other studies (National Health Commission of the People's Republic of China, 2023; Ganesh et al., 2019). This discrepancy may reflect the complex relationship between *in vitro* chemosensitivity and considerations of long-term *in vivo* efficacy and toxicity, underscoring the need for caution when translating PDTO findings to guide clinical decisions.

HER2-positive CRC is often more aggressive and associated with a relatively poorer prognosis (Chen et al., 2022; Wang et al., 2019). Chemotherapeutic agents such as 5-FU primarily kill tumor cells by inducing DNA damage and apoptosis. However, if HER2 is overactive, these survival pathways may be persistently activated, potentially attenuating chemotherapy-induced apoptosis. A study by Sirui Long et al. found that upregulation of certain HER2-related factors (e.g., ECM1) can activate the PI3K/Akt pathway, thereby conferring resistance to 5-FU in CRC cells (Long et al., 2022). Similarly direct evidence comes from oxaliplatin-resistant colon cancer cell lines established by Pirpour Tazehkand et al., in which significantly elevated HER2 expression levels were found.

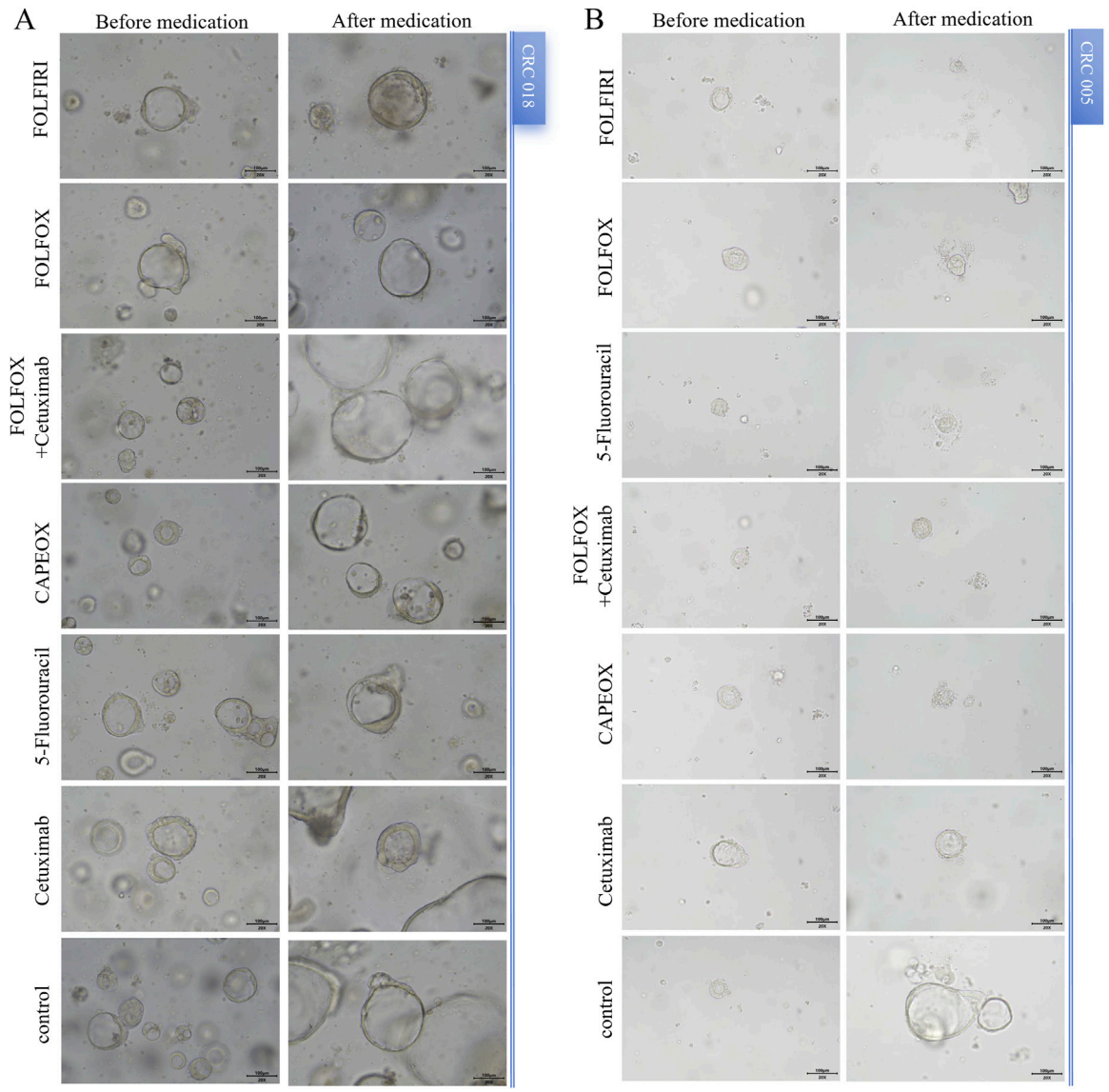

FIGURE 2
Morphological changes in PDOTs before and after drug treatment. **(A)** and **(B)** Representative images of PDTOs treated with different tumor drugs on day 3 of the assay. Case ID was named according to Experimental Specimen Number. Scale bar: 100 μm.

The half-maximal inhibitory concentration (IC50) in these resistant cells increased 7–25-fold compared to parental cells. Furthermore, treatment of resistant cells with a HER2 inhibitor, leading to HER2 downregulation, significantly restored sensitivity to oxaliplatin and increased chemotherapy-induced cytotoxicity and apoptosis rates (Pirpour Tazehkand et al., 2018). In this study, at the organoid level, we revealed an association between low HER2 expression status (1+ vs. 0) and response grades for FOLFIRI and FOLFOX regimens ($P < 0.05$). This finding provides some support for the notion that HER2 overexpression can reduce sensitivity to certain chemotherapeutic agents by promoting survival signaling and altering cellular phenotype.

However, at the population level, multiple studies have not observed HER2 status to significantly alter chemotherapy efficacy or significantly impact patient survival outcomes after receiving chemotherapy (Jang et al., 2023; Richman et al., 2016). The observed disparity between findings at the organoid/cellular level and the patient level is likely multifactorial. Clinically, true HER2 amplification is rare (~2–5%) and spatially heterogeneous; large adjuvant and metastatic trials therefore contained too few HER2-positive cases to detect small differences in response to standard fluoropyrimidine-, oxaliplatin- or irinotecan-based regimens (Richman et al., 2016; Battaglin et al., 2024). *In vitro*, oxaliplatin- or 5-FU-selected single-clone CRC cell lines carry very high HER2 copy numbers and face constant drug exposure, so the HER2-Akt/Nrf2 survival axis alone is sufficient to block apoptosis-conditions that do not recapitulate the fluctuating drug levels, immune attack and stromal barriers present in patients (Pirpour Tazehkand et al., 2018; Jang et al., 2023). Finally, tumour-derived CAF exosomes carrying miR-92a-3p and other effectors induce cross-resistance to 5-FU and oxaliplatin in both HER2-negative and -positive cells, biologically masking any HER2-specific contribution at the population level (Hu et al., 2019).

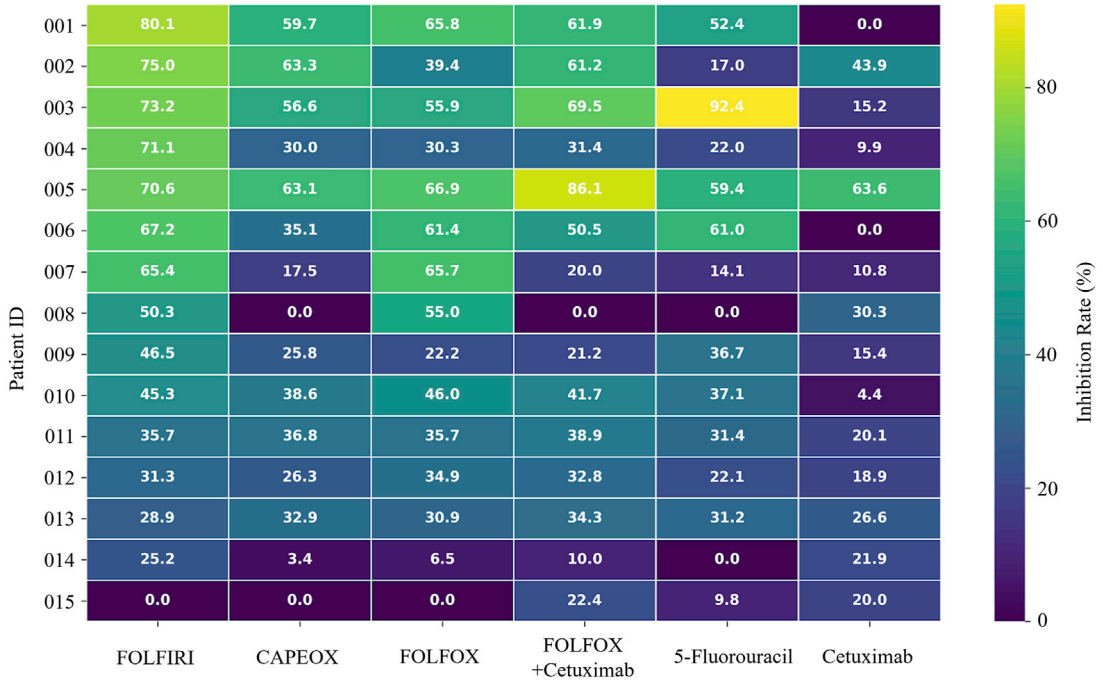

FIGURE 3
Heatmap of drug inhibition rates (%).

TABLE 1 The rates (%) of inhibition grade corresponding to different drugs.

Test drug	A (%)	B (%)	C (%)	D (%)
5-Fluorouracil	6.7	20	26.7	46.7
FOLFIRI[a]	33.3	26.7	20	20
CAPEOX	0	26.7	26.7	46.7
FOLFOX	0	40	40	20
Cetuximab	0	6.7	13.3	80
FOLFOX + Cetuximab	6.7	26.7	33.3	33.3

[a]For the FOLFIRI, regimen, the percentages of subjects achieving inhibition rates of Grade A, B, C, and D were 33.3%, 26.7%, 20%, and 20%, respectively. Furthermore, the percentage of subjects achieving Grades A and B combined was the highest among all regimens, at 60%.

TABLE 2 Stratified analysis of inhibition grade by HER2 status for different drug regimens.

Statistical indicators	5-Fluorouracil	FOLFIRI	CAPEOX	FOLFOX	Cetuximab	FOLFOX+ Cetuximab
U	17.0	7.5	11.5	2.5	16.0	7.0
P^a	0.265	0.029	0.080	0.004	0.152	0.024

[a]Statistical significance was defined as $P < 0.05$.

More prospective studies (e.g., stratified analysis of chemotherapy efficacy based on HER2 status), as well as in-depth molecular mechanistic studies, are warranted to fully elucidate the role of HER2 in CRC chemotherapy sensitivity and optimize individualized treatment strategies.

Conclusion

This study provides a detailed view of the chemosensitivity landscape in a cohort of CRC PDTOs, demonstrating their potential as a preclinical drug screening model. Furthermore, this

study suggests that HER2 status is a factor influencing the sensitivity of colorectal cancer to conventional chemotherapeutic agents, but may not be a strong determinant.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Medical Ethics Review Committee/Second People's Hospital of Jiangyou. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from primarily isolated as part of your previous study for which ethical approval was obtained. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

JaL: Conceptualization, Writing – original draft, Investigation. DL: Data curation, Writing – original draft. JnL: Formal Analysis, Methodology, Writing – review and editing. WF: Investigation, Resources, Writing – review and editing. QJ: Data curation, Writing – review and editing. YB: Formal Analysis, Writing – review and editing. AH: Visualization, Writing – review and editing. FC: Conceptualization, Funding acquisition, Writing – review and editing.

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Conflict of interest

Authors JnL and YB were employed by Chengdu Tianfu Organoid Biobank Co., LTD.

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A diaryl urea derivative, SMCl inhibits cell proliferation through the RAS/RAF/MEK/ERK pathway in hepatocellular carcinoma

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Introduction: Hepatocellular carcinoma (HCC) ranks among the three most prevalent cancer-related diseases in terms of incidence. Hence, exploring drugs for HCC therapy is of great significance. Compounds with a diaryl urea structure have been reported to exhibit a broad range of biological activities, including anticancer activity. This study focuses on the specific diaryl urea derivative 4-(4-(3-(2-chloro-3-(trifluoromethyl)phenyl)ureido)phenoxy)-N-methylpicolinamide (SMCl), with particular emphasis on investigating its therapeutic effects against hepatocellular carcinoma (HCC) and elucidating the underlying molecular mechanisms.

Methods: *In vitro* anti-cancer effects of SMCl were evaluated in HCC cell lines using MTS, colony formation, and wound healing assays. Western blot analyzed RAS/RAF/MEK/ERK pathway modulation. *In vivo* efficacy was assessed using a xenograft model.

Results: The MTS and colony formation assays demonstrated that SMCl significantly decreased the viability of HCC cells. Western blot analysis demonstrated that SMCl effectively suppressed hepatocellular carcinoma proliferation by markedly inhibiting the RAS/RAF/MEK/ERK signaling pathway, with this inhibitory effect exhibiting both time- and concentration-dependent characteristics. SMCl also demonstrated significant therapeutic efficacy in the xenograft tumor model, achieving a tumor inhibition rate of 72.37%. Notably, it showed no significant impact on spleen weight or body weight in mice, indicating low toxicity to normal tissues.

Conclusion: This study first elucidates the effects of SMCl on HCC cells and its impact on the RAS/RAF/MEK/ERK signaling pathway, providing a potential active compound for the clinical treatment of liver cancer.

KEYWORDS

hepatocellular carcinoma, diaryl urea derivative, cell proliferation, RAS/RAF/MEK/ERK, bioactive compound

1 Introduction

At present, hepatocellular carcinoma (HCC) ranks as the fourth leading cause of cancer-related mortality globally. Especially in less developed regions of Africa and Asia, the death toll attributed to this disease is predicted to rise sharply in the next decade (Siegel et al., 2024). Chronic infections caused by hepatitis B or C virus, nonalcoholic steatohepatitis due to obesity (with or without type 2 diabetes), and excessive alcohol consumption are key risk factors for HCC (Alqahtani et al., 2019; Reddy et al., 2023). Additional risk factors encompass metabolic disorders like α1-antitrypsin deficiency, hemochromatosis, and autoimmune conditions. The intake of aflatoxin-contaminated foods and tobacco usage also have a notable impact on the progression of this disease. The variation in risk exposure is broad, depending on geographic and sociocultural contexts (Ducreux et al., 2023). Despite available surgical and transplantation approaches that offer the best outcomes, only about 15% of patients are eligible for these potentially life-saving measures, as the majority are diagnosed at advanced stages of the disease (Anwanwan et al., 2020; Terrault et al., 2023).

Chemotherapy remains one of the primary treatment modalities for HCC, mainly due to its significant effectiveness in rapidly diminishing tumor burden. HCC is often characterized by large tumor masses or concurrent liver dysfunction, which makes patients ineligible for curative approaches such as surgery, radiotherapy, or immunotherapy. In such cases, chemotherapy employs cytotoxic agents to swiftly kill cancer cells or inhibit their proliferation, leading to substantial tumor shrinkage and alleviation of clinical symptoms, thereby creating opportunities for subsequent treatments (Kew, 2011). Moreover, chemotherapy plays a crucial role in various combination therapy regimens, such as those involving targeted therapies or immunotherapies, enhancing overall therapeutic efficacy. Despite its limitations, including toxicity and the emergence of drug resistance, chemotherapy remains indispensable in the management of HCC, particularly in settings where therapeutic resources are constrained, or alternative options are unavailable. Chemotherapeutic agents are affordable and have broader applicability, making them especially vital for patients in low- and middle-income countries (Ocran Mattila et al., 2021). Introduced in 2007 for treating advanced inoperable HCC, Sorafenib, a multikinase inhibitor that halts cell proliferation and angiogenesis, represented a breakthrough (Kim et al., 2017). Subsequently, other multi-kinase inhibitors like Lenvatinib have received approval and are found to be equally effective as Sorafenib for initial treatment. Drugs like Regorafenib, Cabozantinib, and Ramucirumab have been approved as secondary agents to increase survival rates. Although new immunotherapies for HCC have offered new hope, their overall effectiveness is still limited (Yang et al., 2023; Bicer et al., 2023). Identifying novel candidate compounds suitable for clinical development in HCC management persists as a vital research imperative.

Diphenyl urea derivatives possess significant application potential due to their unique structure and diverse biological activities. Structurally, the core of diphenyl urea derivatives consists of a urea group (-NH-CO-NH-) linking two aromatic rings (Ladd et al., 2024). Various substituents (such as halogens, alkyl groups, amino groups, etc.) can be attached to the aromatic rings, allowing for modulation of the molecule's electronic effects and spatial configuration, which in turn affects its biological activity and selectivity. This structural characteristic endows the diphenyl urea derivatives with enhanced molecular stability and chemical tunability, facilitating the optimization of their pharmacological properties through structural modifications. Numerous diphenyl urea derivatives have been identified for their anticancer activity, with multi-kinase inhibitors being a prominent example. Sorafenib, a multi-kinase inhibitor, effectively inhibits tumor cell proliferation and angiogenesis, showing significant therapeutic effects in liver and kidney cancers.

Additionally, it has been reported that Sorafenib suppresses the growth and metastasis of hepatocellular carcinoma by blocking the activation of STAT3. Furthermore, certain diphenyl urea derivatives have demonstrated inhibitory effects on viral protein targets, including SARS-CoV-2 and influenza A virus (IAV) (Degirmenci et al., 2020). These derivatives interact with specific proteins involved in viral replication, reducing viral load and slowing down the disease progression. In the research of SARS-CoV-2, it has been found that diphenyl urea derivatives can inhibit the main protease (Mpro) and RNA-dependent RNA polymerase (RdRp), both of which are critical for viral replication. The diverse biological activities of diphenyl urea derivatives have made them a focal point in drug development.

The successful application of Sorafenib highlights the significance of diphenyl urea derivatives in cancer treatment. With ongoing research, more diphenyl urea derivatives with specific biological activities are expected to be discovered. An important aspect of cell communication is the RAS/RAF/MEK/ERK signaling network, which is responsible for regulating processes like cell proliferation, survival, and differentiation. Several natural agents, including alkaloids, phenolics, terpenoids, and nano-formulations, impact the Ras/Raf/MAPK route (Gravandi et al., 2023). This pathway also manages signals from complex cell networks to regulate cellular activities. Although researchers have known the essential elements of the MAPK pathways for nearly four decades, fully understanding the intricate molecular dynamics that govern these pathways remains a challenge. Intense efforts have been made to control Raf, particularly after identifying drug resistance and unexpected activation upon the attachment of inhibitors to the kinase (Imoto et al., 2023). Phosphorylation and conformational changes, such as autoinhibition and dimerization, are involved in Raf regulation. A few RAS/RAF/MEK/ERK route inhibitors have demonstrated potential in the treatment of liver cancer in clinical trials (Spencer-Smith and Morrison, 2024; Zinatizadeh et al., 2019). As a cancer treatment strategy, dual inhibition of this pathway is highly effective (Cheng et al., 2019).

In this study, we have discovered a new active compound, 4-(4-(3-(2-chloro-3-(trifluoromethyl) phenyl) ureido) phenoxy)-N-methylpicolinamide, which bears structural resemblance to sorafenib. This compound shares the aminoquinoline backbone, an aromatic ring, and fluorine atoms with sorafenib. The aromatic ring provides hydrophobicity, while the fluorine atoms contribute to enhanced membrane permeability and metabolic stability. The key difference is that sorafenib has a chlorine atom at the para-position of the aromatic ring, whereas in 4-(4-(3-(2-chloro-3-(trifluoromethyl) phenyl) ureido) phenoxy)-N-methylpicolinamide, the chlorine atom is situated between the aromatic ring and the trifluoromethyl group. We named this new compound "sorafenib meta-chlorine" and abbreviated

it as SMCl. This study aims to evaluate the efficacy of SMCl, a new RAS/RAF/MEK/ERK inhibitor, in treating HCC through cell and animal model experiments.

2 Materials and methods

2.1 Materials

2.1.1 Cell culture

The Huh7, Hep3B, PLC/PRF/5 and SMMC7721 cell lines were cultured in DMEM (Dulbecco's Modified Eagle Medium, Gibco, United States) supplemented with 10% fetal bovine serum (ExCell Bio, China) and 1% penicillin-streptomycin solution (100 U/mL each, CellorLab, China). SMMC7721 cell line was purchased from the American Type Culture Collection (ATCC). Hep3B, PLC/PRF/5 and Huh7 cell line were obtained from HyCyte (https://www.hycyte.com/; Catalogue No. TCH-C195, No. TCH-C298 and No. TCH-C217). The culture medium was kept at 37°Cin an incubator with 5% carbon dioxide (Ding et al., 2015).

2.1.2 Chemicals

DMSO (Sigma-Aldrich, United States) was used to dissolve SMCl, which was supplied by the QCS Reference Material Research and Development Center in China. We conducted a qualitative analysis of SMCl, and the results are shown in the Supplementary Figures S1–S8. Sorafenib (Shanghai Macklin Biochemical, China); Antibodies against Ras, Raf, pRaf, and GAPDH(Abcam, United Kingdom); Rabbit anti-mouse IgG-HRP conjugates, p21, Erk, and pErk (Cell Signaling Technology, United States); All antibodies were utilized for Western blot analysis. The [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium(MTS) and phenazine methosulfate(PMS) were provided by Promega Corporation in the US. The MTS and PMS powders are individually dissolved in Dulbecco's Phosphate-Buffered Saline (DPBS), sterilized, and stored at −80°C. Beyotime Biotechnology in China is responsible for the production of the EdU Cell Proliferation Kit.

2.2 Methods

2.2.1 Evaluation of cell viability

Following the methodology described in a previous study (Wu et al., 2015), the MTS was applied to determine the viability of the cells during each experiment, Hep3B and Huh7 cells were seeded in 96-well flat-bottom plates at an appropriate concentration. The next day, the cells were subjected to a range of different concentrations of SMCl. Simultaneously, the control cells were given the same amount of vehicle (DMSO) as the cells that were treated with the drug. Following a treatment period of 72 h, Remove the compound-containing medium and replace it with the prepared solution (MTS:PMS ratio of 20:1), adding 100 μL per well to a 96-well plate. Incubate for 1–4 h, then determine the optical density at 490 nm.

2.2.2 EdU test

Twenty-four well plates were used to seed the cells. Following the natural adhesion process, different concentrations of SMCl were added, specifically 0 μM, 2.5 μM, 5 μM, and 10 μM. The EdU test was utilized in accordance with the instructions supplied by the kit to determine the number of cells in the proliferation phase.

2.2.3 Formation of colonies

The cell lines Hep3B and PLC/PRF/5 were cultured for a period of 48 hours after being placed into 12-well plates at a total of one thousand cells in each well. After that, the cells were subjected to SMCl over a period of 24 hours, with concentrations of 2.5, 5, and 10 μM. To encourage the formation of colonies, the cells were cultured for a span of 10 days, with medium changes occurring every 5 days. Finally, photographs were taken of the colonies (Luo et al., 2023).

2.2.4 Wound healing test

In order to evaluate cell locomotion, the wound closure assay was utilized *in vitro*, as outlined in the previous section (Zhang et al., 2019). Every single experiment was carried out three times, and each experiment was replicated three times to ensure accuracy.

2.2.5 Analysis of the western blot

Cells were transplanted into plates with six wells. Following 48 h of treatment with SMCl at graded concentrations (0, 2.5, 5, and 10 μM), cells were harvested for subsequent analysis. Another group of cells was treated with 10 μM SMCl for varying durations (0, 24, 36, and 48 h) before collection. The cells were lysed with RIPA lysis buffer that contained PMSF (Beyotime, China). Protein quantification was performed using a BCA assay kit (Beyotime, China). Proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Canada). Membranes were blocked with 5% skim milk at room temperature for 1 h. After that, the membranes were subjected to an incubation procedure using primary antibodies that targeted Erk, pErk, and GAPDH (Cell Signalling Technology, Danvers, Massachusetts, United States), which was then followed by the application of secondary antibodies that were appropriate for the situation. The use of enhanced chemiluminescence (ECL) detection reagents allowed for the visualization of protein bands (Zhang et al., 2023).

2.2.6 The study of animals

The development of a subcutaneous tumor model was carried out in order to gain an understanding of the effects that SMCl has on the growth of tumors in living organisms (Kim et al., 2019). The Guangdong Provincial Medical Experimental Centre provided the female BALB/c mice that were used in this study. These mice were aged between four and 5 weeks and were housed in the SPF Laboratory Animal Centre of Shenzhen Rongwan Biotechnology Co., Ltd. When carrying out procedures that involved animals, the Rongwan Biological Laboratory Animal Center strictly adhered to the regulations that had been established for the welfare of laboratory animals and ethics concerning laboratory animals with the reference approval number RW-IACUC-24-0022. SMMC7721 cells, with a concentration of 1×10^6, were administered to each mouse through a subcutaneous injection. The injection was administered in the right flank of each mouse. Beginning on the day that the cell injection was administered, measurements of the body weight and the volume of the tumor were taken every 3 days. For determining the volume of the tumor,

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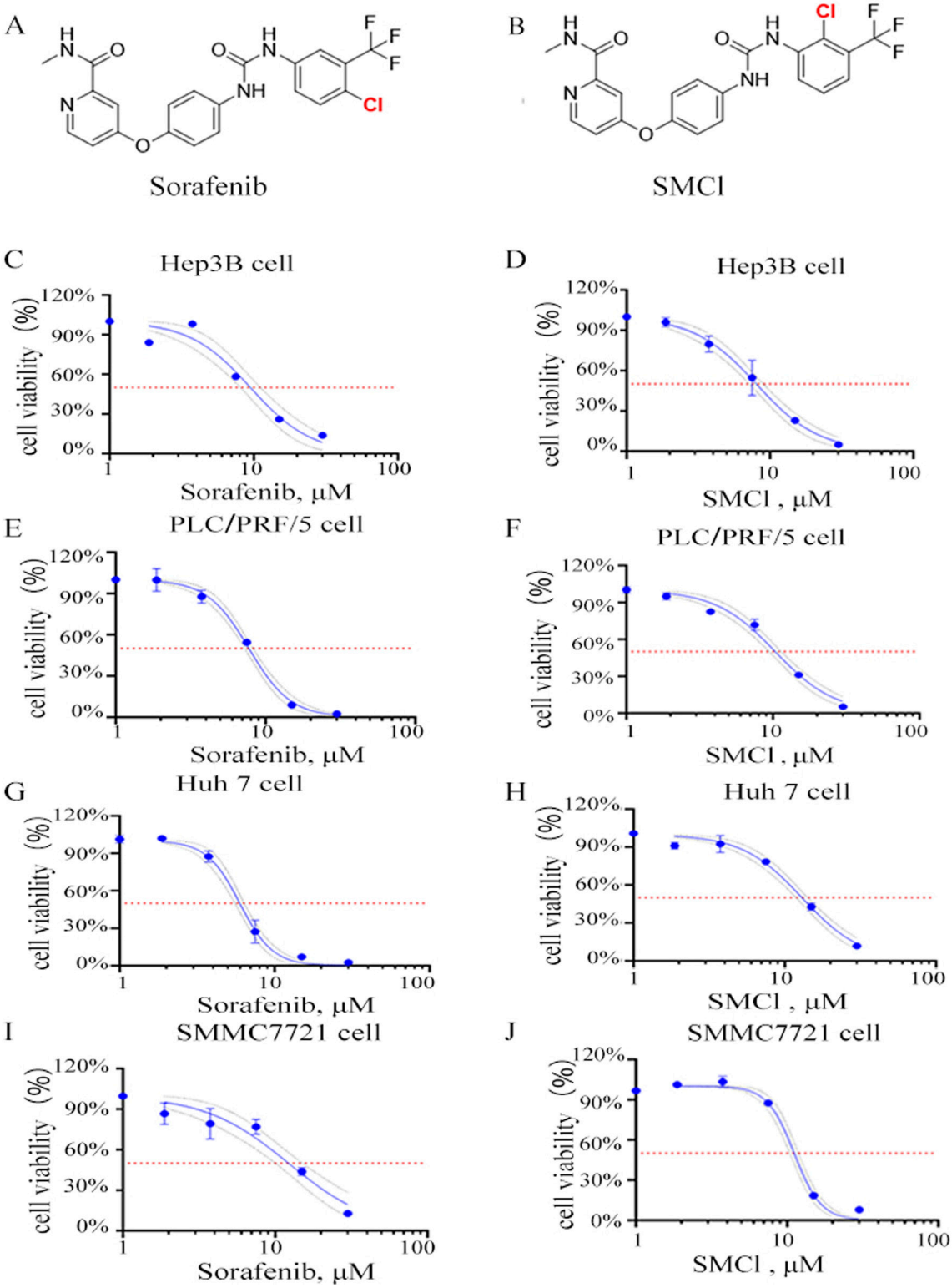

FIGURE 1
Effects of SMCl on the cell viability of HCC cells. **(A,B)** Chemical structure of SMCl and Sorafenib, **(C–J)** Effects of different concentrations of SMCl and Sorafenib on the proliferative ability of liver cancer cells at 72 h.

the formula that was utilized was (length × width^2)/2. The mice were randomly divided into two groups, each consisting of five to seven mice, once the tumors had grown to a volume of 50 mm squared. This was done after the tumors had begun to grow. When cells were administered, the average amount of time it took for tumors to reach a volume of 50 mm cubic was approximately five to 6 days, with a

standard deviation of three and a half percentage points. Included in the treatment groups were both the SMCl (50 mg/kg/day) group as well as the control group, which consisted of the drug vehicle. The administration of each treatment was carried out by means of oral gavage. Following the completion of the experiment, which lasted for a period of 3 weeks, the tumors were promptly removed after the euthanasia procedure was completed. Each tumor was dissected to obtain tumor tissue lysates for Western blot analysis. This was done to make the histological examination more manageable.

2.2.7 Statistical analysis

The quantitative findings were obtained from at least three distinct trials, and the data is provided as the average value together with the standard deviation. The statistical analyses were conducted using GraphPad Prism Software, version 7.0 (GraphPad Inc., La Jolla, California, United States). We employed the student's t-test to ascertain significant disparities between two groups. To evaluate the variation among various groups, either one-way or two-way ANOVA was utilized, along with Bonferroni's correction for multiple comparisons.

3 Results

3.1 SMCl possesses substantial anti-proliferation effects on HCC cell lines

The structure of SMCl and its differences from sorafenib are shown in Figures 1A. Supplementary Figure S1 shows that the purity of SMCl is 98% as determined by HPLC. Supplementary Figures S2, S3 represent the ^{1}H NMR and ^{13}C NMR spectra of SMCl (Figures 1A,B). To further distinguish SMCl from sorafenib, we performed two-dimensional NMR analysis of SMCl, with Supplementary Figures S4–S8 presented respectively. To determine the potential inhibitory effect of SMCl on liver cancer cell growth, we first assessed its influence on cell proliferation and viability using the MTS assay in PLC/PRF/5 and SMMC7721 cells, using sorafenib as the positive control compound. As depicted in Figures 1C–J, SMCl demonstrated a concentration-dependent reduction in cell viability across Hep3B, PLC/PRF/5, and SMMC7721 cell lines. The IC_{50} values of SMCl for Hep3B, PLC/PRF/5, Huh7 and SMMC7721 cells were 8.033 μM, 10.37 μM, 12.98 μM and 11.83 μM, respectively and the IC_{50} values of sorafenib for Hep3B, PLC/PRF/5, Huh7 and SMMC7721 cells were 9.477 μM, 7.755 μM, 5.697 μM and 12.22 μM, respectively. These results indicate that SMCl exhibits inhibitory effects on different types of liver cancer cells. Specifically, the lowest IC_{50} value (8.033 μM) was observed in Hep3B cells, suggesting that SMCl has the most significant anticancer effect in this cell line and can effectively inhibit cell proliferation at lower concentrations. Although the IC_{50} values for PLC/PRF/5 and SMMC7721 cells are higher, at 10.37 μM and 11.25 μM respectively, these data still demonstrate that SMCl exerts a certain level of inhibition on liver cancer cells. Notably, compared to sorafenib, SMCl exhibits stronger effects on Hep3B and SMMC7721 cells, while the opposite is observed in Huh7 and PLC/PRF/5 cells. These experimental results highlight the broad activity of SMCl across different liver cancer cell lines, providing a theoretical basis for its development as a potential anti-liver cancer drug.

3.2 SMCl inhibits the migrative and colony forming capacities of HCC cells *in vitro*

For determining the effect that SMCl has on the migratory and colony-forming capabilities of hepatocellular carcinoma (HCC) cells, we conducted wound healing and colony-formation assays. The utilization of various concentrations of SMCl was shown to effectively impede the movement of HCC cells, as shown in Figures 2A–D. Based on this observation, it appears that SMCl exerts an effect that is dose-dependent in terms of its ability to inhibit the migration of hepatocellular carcinoma (HCC) cells, and the high concentration (10 μM) inhibited Hep3B colony formation by more than 50%. In addition, the wound healing assay provided further evidence that supports these findings (Figures 2E–J). More precisely, the study found that increased concentrations resulted in a significant reduction in the ability of HCC cells migration, particularly in SMMC7721 cells, this inhibitory effect on wound closure was more pronounced, showing over 60% inhibition at 36 h, as evidenced by SMCl. The results emphasize the ability of SMCl which has the capability to inhibit the migration and colony formation of HCC cells, suggesting that it may suppress the progression of hepatocellular carcinoma by affecting cell motility and proliferation. The development of HCC is not only dependent on the proliferation of tumor cells but also closely related to their migration and invasion capabilities. Cell migration, especially during cancer metastasis, is a key factor in determining tumor spread (Li et al., 2023). Therefore, the inhibitory effect of SMCl may indicate that it can interfere with the invasive behavior of tumor cells, limiting the spread of cancer cells to surrounding tissues and thus reducing the risk of liver cancer metastasis.

3.3 SMCl inhibits the growth of HCC cells via the RAS/RAF/MEK/ERK pathway

For the EdU experiment, we used Hep3B Cell lines divided into a control group and an SMCl treatment group (10 μM). After 48 h of treatment, fluorescence microscopy indicated that the SMCl treatment group had a much smaller number of EdU-positive cells than the control group (Figure 3A). These results indicate that SMCl can significantly inhibit DNA synthesis activity in Hep3B cells, suggesting its potential anti-tumor effect through inhibition of cell proliferation. Moreover, treatment of Hep3B and PLC/PRF/5 cells with increasing concentrations of SMCl resulted in dose-dependent downregulation of Ras, phosphor-Raf, and Erk protein levels (Figure 3B). Notably, SMCl reduced both phosphorylated and total forms of Raf and Erk, suggesting non-specific inhibition of the MAPK pathway. Time-course experiments demonstrated progressive decreases in Erk and Raf phosphorylation with prolonged SMCl exposure (Figure 3C), confirming time-dependent suppression of pathway activity. These findings collectively indicate that SMCl effectively inhibits the RAS/RAF/

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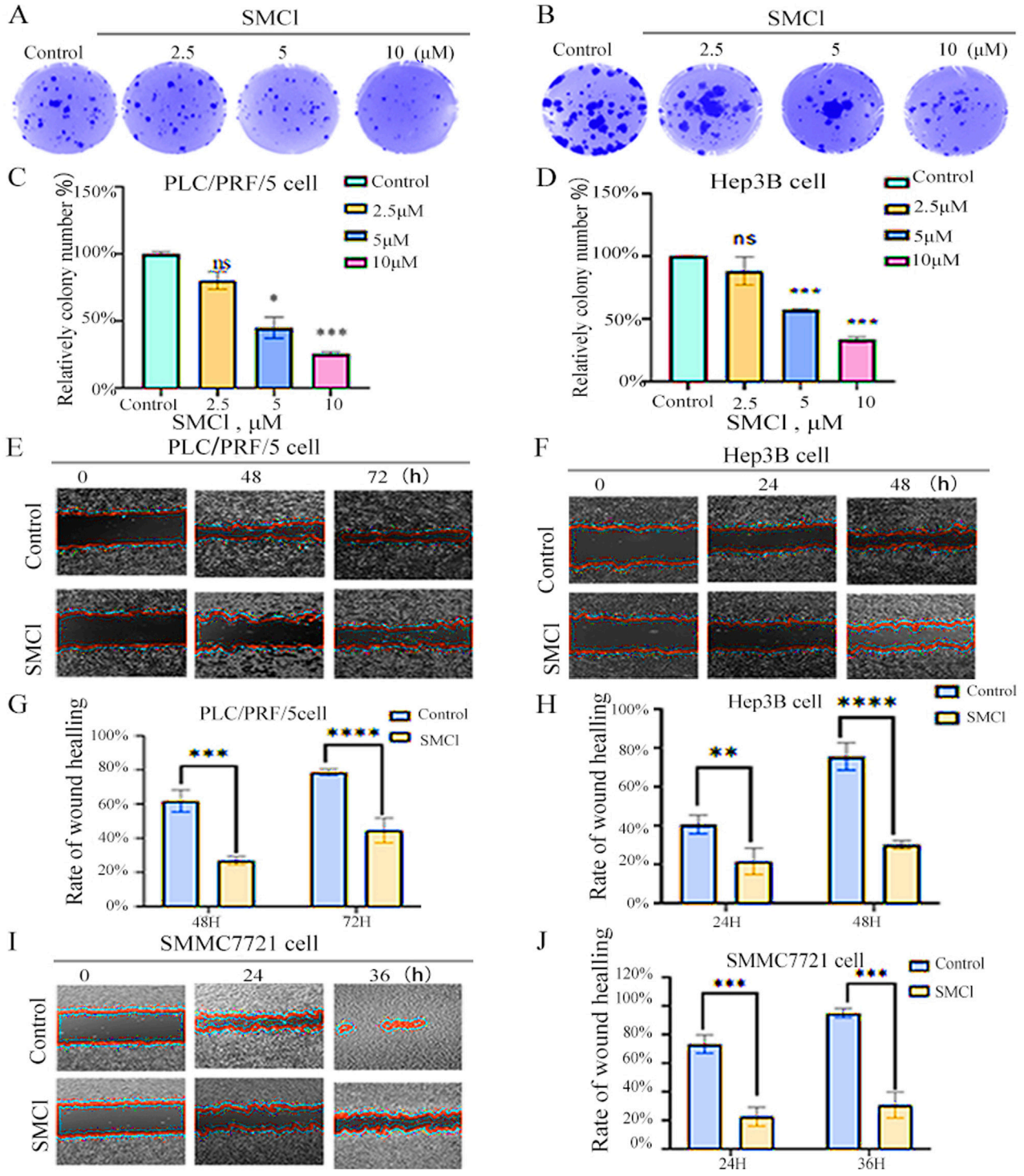

FIGURE 2
SMCl suppresses cell migration and colony formation. **(A,B)** Cells were treated with 0, 2.5, 5, and 10 μM SMCl for the indicated time. **(C,D)** Relative colony number at different concentration of SMCl. **(E,F,I)** Wound healing assay was performed. Cells were treated with 10 μM SMCl at 0, 24, 36, and 48 h. Scale bar, 100 μm. Experiments were independently conducted in triplicate. **(G,H,J)** The area of non-migrant cells was quantified using ImageJ software. Data are presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. the control group).

MEK/ERK signaling cascade through both concentration- and duration-dependent mechanisms. Concurrently, examination of the downstream proliferation-associated protein p21 revealed significant upregulation following SMCl treatment, mechanistically substantiating its anti-proliferative efficacy through cell cycle checkpoint activation.

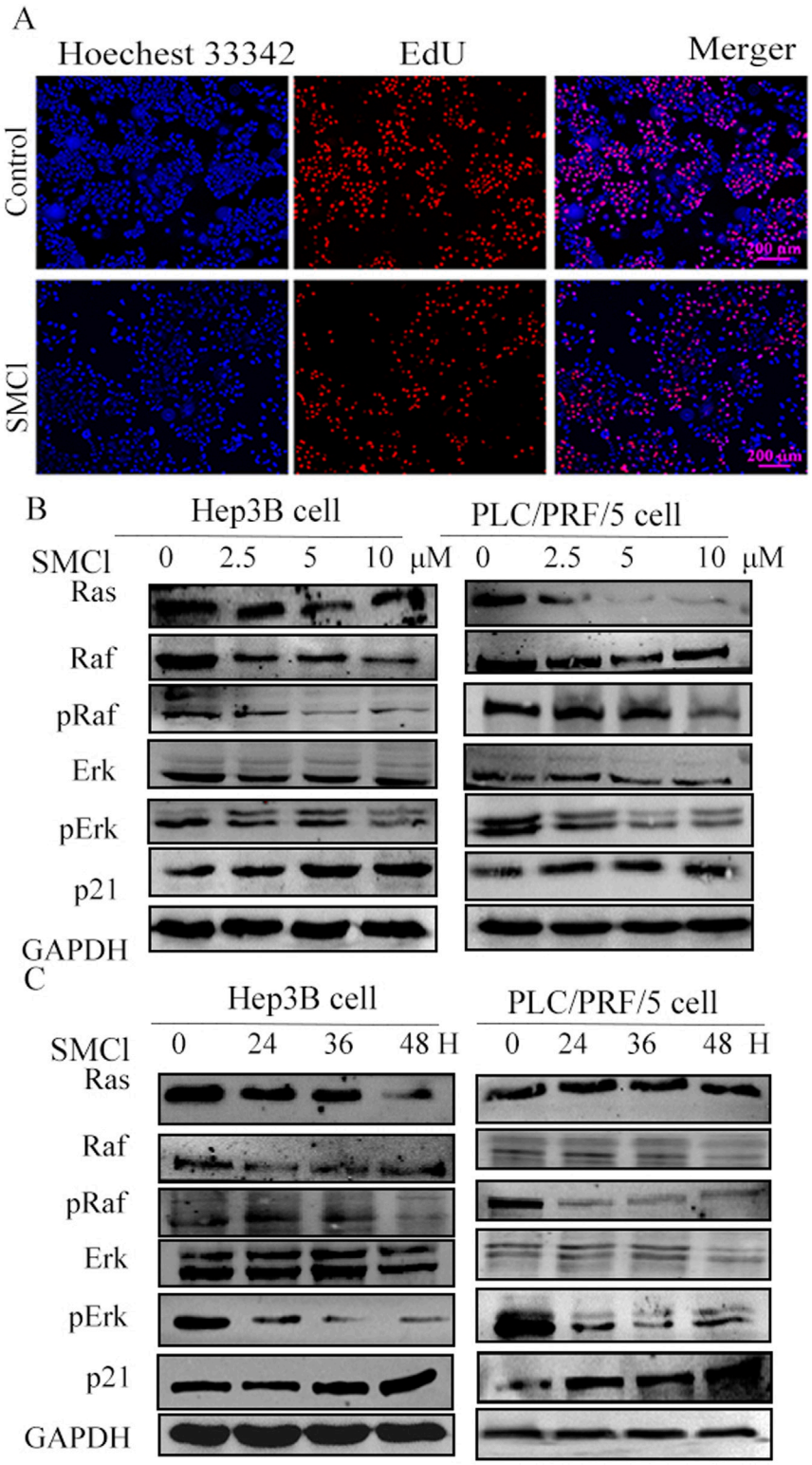

FIGURE 3
Investigated the impact of SMCl on the Ras/Raf/MAPK pathways in HCC cell lines. **(A)** Hep3B cells were divided into a control group and an SMCl treatment group (10 μM). After 48 h of treatment, fluorescence microscopy showed a marked reduction in the number of EdU-positive cells in the SMCl-treated group compared to the control group. **(B)** Effects of 0, 2.5, 5, and 10 μM SMCl treatment for 48 h on protein levels of Ras, Raf, Erk, and p21 in both Hep3B and PLC/PRF/5 cell lines. **(C)** Time-dependent effects of 10 μM SMCl treatment at 0, 24, 36, and 48 h on protein levels of Ras, Raf, Erk, and p21 in Hep3B and PLC/PRF/5 cells.

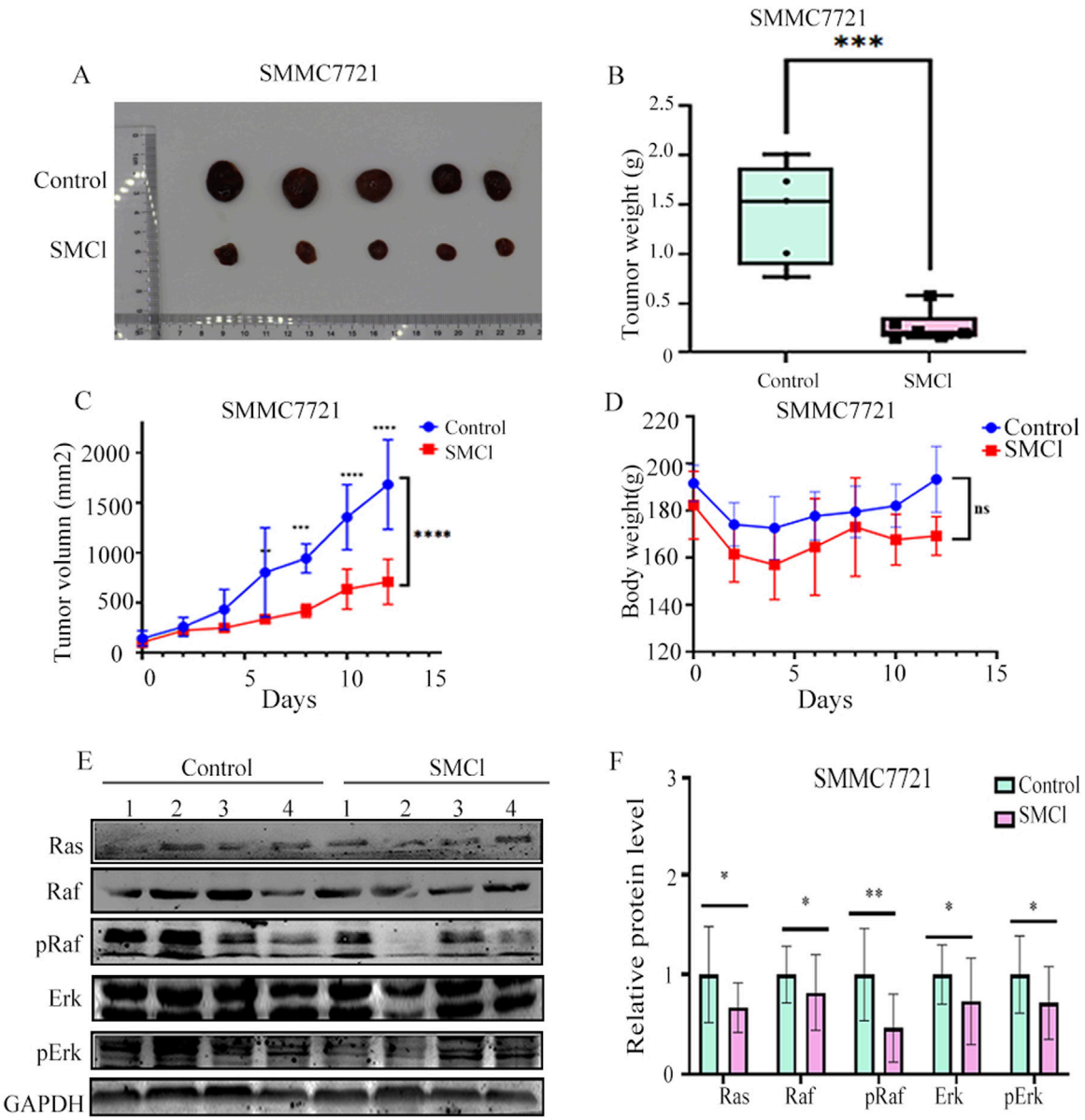

FIGURE 4
SMCl inhibits tumor progression in the SMCC7721 xenograft model. **(A)** Nude mouse bearing xenografted tumors were randomly divided into two groups: one as the control group treated daily with DMSO, and the other treated with SMCl 50 mg/kg/day for 12 days. Dissected tumors were obtained from mice in both the control and SMCl groups. **(B)** Tumor weight of subcutaneous tumors from the control and SMCL groups. **(C)** Comparison of subcutaneous tumor volumes from the control and SMCL groups (n = 5). **(D)** Comparison of body weight of nude mice from the control and SMCL groups. **(E,F)** Protein levels of pRaf, pErk, and Ras in subcutaneous tumors from the DMSO and SMCL groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3.4 SMCl inhibits tumor growth in xenograft mouse models

We investigated whether SMCl could reduce tumor growth in a CDX mice model. As demonstrated in Figures 4A–C, SMCl (50 mg/kg) significantly inhibited the growth of the subcutaneous SMMC7721 tumor and compared to the control group, the tumor inhibition rate reached 72.37%. Notably, body weight was not significantly different between the SMCl group and the control group mice (Figure 4D). Western blotting revealed the same trend of associated protein changes (Figures 4E,F). The results of all animal experiments indicate that SMCl can effectively inhibit tumor growth while having no significant effects on the animals themselves, indicating low toxicity and side effects, which suggests good biological safety. Low toxicity is a critical feature in cancer treatment, as it reduces damage to normal tissues and enhances the safety and tolerability of treatment (Lou et al., 2015). Additionally, through protein extraction from animal tissues and Western blot experiments, the study also confirmed the effect of SMCl on the RAS/RAF/MEK/ERK signaling pathway. This pathway plays a key role in the development of liver cancer and other tumors by regulating processes such as cell proliferation, differentiation, and

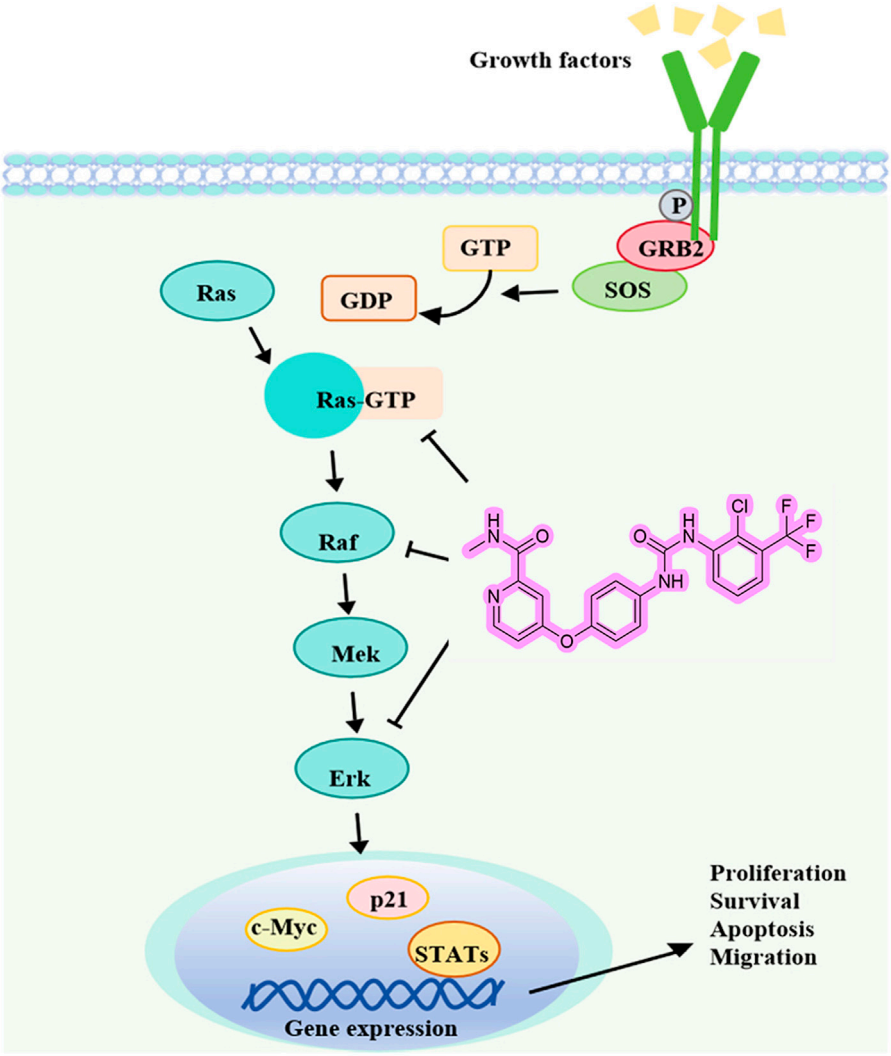

FIGURE 5
SMCl exerts anti-HCC effects by targeting the RAS/RAF/MEK/ERK pathway.

migration, promoting tumor growth and metastasis. Therefore, Impact of SMCl on this pathway further suggests that it may inhibit tumor progression by interfering with critical signaling pathways in tumor cells. These animal study results provide strong evidence of SMCl biological activity *in vivo* and support its potential as a therapeutic agent for liver cancer.

4 Discussion

Liver cancer is characterized by high mortality and incidence rates, while the scarcity of clinical therapeutics remains a persistent challenge. The discovery of potential candidate compounds is therefore of critical importance. With diaryl urea as the core of anticancer molecules, researchers can develop more potent drugs that precisely attack cancer cells while leaving healthy cells unharmed (Wang et al., 2020; Liu et al., 2016). SMCl is a diaryl urea derivative, and this study represents the first evaluation of its antitumor effects on Hep3B and SMMC7721 liver cancer cells, demonstrating its potential as a promising therapeutic agent. These initial findings have been further supported by subsequent cell and animal experiments, providing solid evidence of SMCl efficacy in the treatment of HCC. Furthermore, our study revealed that SMCl exhibits the characteristic biological activity of diaryl urea derivatives, effectively inhibiting the activity of several key intracellular kinases, including RAF, RAS, and ERK kinases. The mechanism of SMCl is illustrated in Figure 5.

RAF and ERK kinases play critical roles in tumor cell proliferation, migration, and angiogenesis, with aberrant kinase activity being a major driving force in tumor development and progression (Garuti et al., 2016). By inhibiting multiple kinase activities, SMCl exerts its antitumor effects through the RAS/RAF/MEK/ERK signaling pathway (Bahar et al., 2023). As a consequence, the primary signaling pathway targeted by multi-kinase inhibitors is the mitogen-activated protein kinase (MAPK) pathway, specifically the RAS/RAF/MEK/ERK cascade. Raf kinase plays a crucial role in this pathway by sequentially activating downstream targets like Mek and Erk, regulating essential cellular processes such as development, cell cycle control, multiplication,

specialization, and longevity (Scardaci et al., 2024). An imbalance in this pathway has been tied to several diseases, with cancer being one of them. The story of Raf inhibitors begins with sorafenib, a drug initially aimed at targeting Ras-driven tumors. Unfortunately, it was insufficient as cancer cells developed resistance to the treatment (Wei et al., 2023; Liu et al., 2023). SMCl, as a diaryl urea derivative structurally similar to sorafenib, has demonstrated comparable safety and therapeutic efficacy (Ghannam et al., 2023; Ommi et al., 2023). Interestingly, SMCl is often produced as a byproduct during the manufacturing process of sorafenib. Due to the structural similarity between SMCl and sorafenib, scientists initially expressed concerns that this byproduct might interfere with sorafenib's mechanism of action to some extent, potentially diminishing its efficacy. In cancer treatment, the potency and specificity of a drug are critical to its clinical outcomes, which made this potential impact a matter of widespread attention. However, our study revealed findings that were contrary to these initial concerns—SMCl not only did not compromise sorafenib's efficacy but also demonstrated biological activity similar to that of sorafenib. This discovery suggests that SMCl exerts its anticancer effects through mechanisms akin to those of sorafenib, inhibiting the proliferation and migration of tumor cells. Furthermore, as a byproduct of sorafenib production, SMCl has the advantages of being relatively cost-efficient and having a stable supply, which provides significant economic and technological benefits for its future large-scale production and drug development. In summary, these findings not only offer new insights into the mechanism of action of sorafenib but also lay a solid foundation for the development of novel anticancer drugs with enhanced efficacy and reduced side effects.

However, our study has certain limitations that need to be addressed in future research. Firstly, although experimental results indicate that SMCl effectively reduces Ras expression, its precise molecular mechanism remains unclear. It is still unknown whether SMCl directly binds to Ras to inhibit its activity or indirectly regulates upstream or downstream signaling molecules to achieve this effect. Addressing this question will not only help elucidate the mode of action of SMCl but also provide insights for optimizing its structure. Secondly, our current evaluation of SMCl antitumor effects is primarily based on HCC cell lines and xenograft tumor models, resulting in a relatively narrow scope of the research. HCC is a highly heterogeneous disease, with tumors in different patients exhibiting significant differences in genetic mutation profiles, microenvironments, and drug sensitivities (Suresh and Dhanasekaran, 2022; Safri et al., 2024). Beyond this, sorafenib resistance has been well documented, our study did not investigate whether SMCl remains effective against sorafenib-resistant strains. However, the differential responses of various HCC cell lines to SMCl versus sorafenib warrant further exploration. Therefore, in subsequent studies, we will conduct a comprehensive comparison of the mechanistic differences between sorafenib and SMCl at deeper molecular levels, thereby exploring potential solutions to sorafenib resistance and incorporate more diverse vivo models, such as patient-derived xenografts or other HCC subtypes, to comprehensively assess SMCl antitumor activity. Additionally, studies should explore the potential of SMCl in combination therapies, such as with existing targeted drugs or immunotherapies (Vanneman and Dranoff, 2012), to evaluate its synergistic effects. Lastly, although our study preliminarily validated the safety and efficacy of SMCl in cell and animal models, clinical trials are a critical step in translating it into clinical applications. Future clinical trials need to evaluate not only the efficacy of SMCl in HCC patients but also its pharmacokinetic properties, safety, and tolerability. Furthermore, studies should investigate the therapeutic effects of SMCl in patients at different stages of liver cancer and explore its compatibility and applicability with existing treatment regimens.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by Shenzhen Ronwan Laboratory Animal Center. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YF: Software, Investigation, Writing – original draft, Validation, Formal Analysis, Data curation, Writing – review and editing, Conceptualization, Methodology. WF: Data curation, Writing – review and editing. FQ: Writing – review and editing, Software, Methodology, Conceptualization, Supervision. JL: Writing – review and editing, Formal Analysis, Conceptualization. YX: Formal Analysis, Writing – review and editing, Methodology, Software, Investigation. BC: Writing – review and editing, Project administration, Methodology. YL: Resources, Project administration, Writing – review and editing, Methodology, Writing – original draft. XZ: Methodology, Writing – review and editing, Writing – original draft, Funding acquisition, Project administration, Resources.

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Conflict of interest

Authors FQ, JL, YX, and BC were employed by Shenzhen ChemStrong Scientific Co., Ltd. Author YL was employed by Shenzhen Jiangchuan Pharmaceutical Technology Co., Ltd.

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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2025.1605515/full#supplementary-material

SUPPLEMENTARY FIGURE S1
High-Performance Liquid Chromatography (HPLC) chromatogram of SMCl, with a retention time of 8.003 min and a purity of 98%.

SUPPLEMENTARY FIGURE S2
Proton Nuclear Magnetic Resonance (1H NMR) spectrum of SMCl, with chemical shifts and corresponding protons annotated.

SUPPLEMENTARY FIGURE S3
Carbon-13 Nuclear Magnetic Resonance (13C NMR) spectrum of SMCl.

SUPPLEMENTARY FIGURE S4
Distortionless Enhancement by Polarization Transfer (DEPT-135) NMR spectrum of SMCl.

SUPPLEMENTARY FIGURE S5
Correlation Spectroscopy (COSY) NMR spectrum of SMCl, showing proton-proton coupling relationships with cross-peaks indicating correlations.

SUPPLEMENTARY FIGURE S6
Nuclear Overhauser Effect Spectroscopy (NOESY) NMR spectrum of SMCl, displaying spatial correlations between protons, with significant cross-peaks indicating close spatial proximity.

SUPPLEMENTARY FIGURE S7
Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum of SMCl, showing direct one-bond correlations between ^1H and ^13C nuclei.

SUPPLEMENTARY FIGURE S8
Heteronuclear Multiple Bond Correlation (HMBC) NMR spectrum of SMCl, illustrating long-range ^1H-^13C correlations.

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Organoids: their emerging essential role in pathological mechanisms and drug discovery of diabetes and its complications

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Diabetes mellitus is a metabolic disease with a high global prevalence, which affects blood vessels throughout the entire body. As the disease progresses, it often leads to complications, including diabetic retinopathy and nephropathy. Currently, in addition to traditional cellular and animal models, more and more organoid models have been used in the study of diabetes and have broad application prospects in the field of pharmacological research. We summarized the organoid models that have been developed for the study of diabetes mellitus and its complications, and describe their sources, establishment and maturation measures with a focus on pancreatic organoids. For the first time, we summarized the contribution of organoids in diabetes and its complications in terms of mechanism studies, drug screening, and cellular replacement therapies, in the hope of providing a feasible direction for personalized medicine and precision treatment of diabetes and its complications. In addition, we discuss the strengths and limitations of organoids in the field of diabetes and its complications. Nowadays, people strongly advocate personalized medicine and precision medicine, and in this regard, organoid technology has advantages that are unmatched by any conventional experimental models. By combing organoid technology with high-throughput technologies, "patient-specific" drug screening can be achieved faster and more accurately. Organoids are also becoming a potential source of transplantable tissues and functional cell types for cellular replacement therapies in regenerative medicine. With further development of assembly and vascularization techniques, organoids will gradually mature and improve. In conclusion, the 3D organoid system greatly complements the existing modeling system and may play a significant role in future basic and clinical research.

KEYWORDS

diabetes, diabetic complications, organoid, mechanisms research, drug screening, precision treatment

1 Introduction

The history of organoids can be traced back to the 1970s, when primary human skin cells were inoculated with 3T3 cells and the epidermal cells grew from individual cells into colonies, which consisted of keratin-forming cells and eventually formed a stratified squamous epithelium(Rheinwald and Green, 1975; Rheinwatd and Green, 1975). In the 1980s, Bissell et al. found that alveolar-like morphogenesis and the formation of mammary-specific functional differentiation occurred when these cells were cultured on reconstituted basement membranes (Barcellos-hoff et al., 1989; Bissell and Ram, 1989). Sasai eet al. Demonstrated the self-organizing mechanism of local intercellular interactions and found that cortical and retinal neuroepithelial cells still have the potential to build multilayer structures *in vitro* (Eiraku and Sasai, 2012). These examples show that cells have the potential to reassemble and form original organ structures, even if they are completely dissociated.

In this context, the concept of organoids and their application technology emerged. Organoids can be obtained mainly from two types of stem cells: pluripotent embryonic stem (ES) cells and their synthetic induced pluripotent stem cell (iPSC) counterparts, and organ-restricted adult stem cells (ASCs). These are often collectively termed as pluripotent stem cells (PSCs) (Clevers, 2016). Currently, PSCs have been induced to give rise to intestinal, kidney, brain, and retinal organoids, as well as liver tissue. What's more, both brain organoids and retinal organoids (ROs) have shown properties that can recapitulate human organ development, which is not observed in conventional animal models (Lancaster and Knoblich, 2014). Of course, some conditions need to be met for organoids. For example, they must contain multiple cell types of the organs they model and exhibit some function specific to those organs. It is also essential that the organization of the cells be similar to that of the primary organ itself (Lancaster and Knoblich, 2014).

As a technology that is gaining momentum, there are numerous examples which certify the great strengths of organoid technology. They include: the evaluation of drug toxicity; the study of the early stages of disease onset and organ development; and the study of human diseases, such as cancer, rare genetic diseases, and complex multifactorial diseases (Lancaster and Knoblich, 2014; Skardal et al., 2020; Bock et al., 2021). The three-dimensional (3D) structure of cultured cells improves their experimental accessibility compared with traditional animal-only models; moreover, it reduces the number of animals used according to the "3 R" principle (Flecknell, 2002). The significant advantage of organoids over conventional cell cultures is their ability to mimic disease pathology at the organ level (Rossi et al., 2018; Bock et al., 2021). Concurrently, human organoids hold tremendous potential for drug development and precision medicine; furthermore, they provide tractable *in vitro* models that reveal the complex environment of cells (Rossi et al., 2018; Bock et al., 2021). In addition, they hold the promise of contributing to the field of regenerative medicine and cellular replacement therapy by producing transplantable biological structures (Lancaster and Knoblich, 2014; Clevers, 2016; Rossi et al., 2018). Unlike current organ transplants, this treatment avoids immune rejection (Lancaster and Knoblich, 2014). With the development of various modern technologies, organoid methods coupled with single-cell sequencing and spatial profiling technologies may remedy their deficiencies and expand their applications (Bock et al., 2021).

Diabetes mellitus is a metabolic disease characterized by hyperglycemia with inadequate insulin secretion or resistance. Consequently, the prolonged presence of hyperglycemia leads to various tissue dysfunctions, which in turn cause complications such as diabetic retinopathy (DR) and diabetic nephropathy (DN) (Kautzky-Willer et al., 2016; American Diabetes Association, 2020; Ceriello and Prattichizzo, 2021). Therefore, diabetes mellitus is a long-term metabolic disease with multi-organ involvement. Organoids have been used in the study of diabetes and its microangiopathy, and both exposure to hyperglycemia and inflammatory factors *in vitro* and exposure to a diabetic environment in mice resulted in microangiopathy, including thickening of the basement membranes, decreased endothelial to pericyte ratios, and the upregulation of genes such as Angiopoietin 226, Apelin 25, and TNFRSF11B in diabetic organoids (Wimmer et al., 2019b). Hence, the emergence of various organoids provides a usable platform for the study of diabetes. Therefore, we aimed to summarize the organoid models that can be used in the study of diabetes and its complications and describe the applications of these organoids in disease modeling, mechanism studies, and drug screening, as well as their potential for clinical therapeutic applications. Finally, we discuss the shortcomings of organoid technology in current studies of diabetes and its complications, and provide feasible directions for subsequent pharmacological studies and clinical translation. I searched the PubMed database using the keywords "diabetes," "diabetic complications," "diabetic retinopathy," "Diabetic nephropathy," "diabetic kidney disease" in combination with "organoid". We further verify the collected articles to ensure that they are relevant to our topic.

2 Organoid models for diabetes

Diabetes mellitus is a metabolic disease in which multiple organs are involved. Currently, more and more types of organoids are being used in the study of diabetes mellitus and its complications, which we will describe in turn (Figure 1).

2.1 Pancreatic organoids

The development and function of the pancreas undoubtedly play a pivotal role in the development of diabetes. Therefore, we have

Abbreviations: ES, embryonic; iPSC, induced pluripotent stem cell; ASC, adult stem cell; RO, retinal organoid; 3D, three-dimensional; DR, diabetic retinopathy; DN, diabetic nephropathy; EGF, epidermal growth factor; hPSC, human pluripotent stem cell; ECM, extracellular matrix; HUVEC, human umbilical vein endothelial cells; MSC, mesenchymal stem cell; RPC, retinal progenitor cell; IGF1, insulin-like growth factor 1; ACE2, angiotensin-converting enzyme 2; GLP-1, glucagon-like peptide-1; T2DM, type 2 diabetes mellitus; BA, brown adipocyte; PPARγ, peroxisome proliferator active receptor γ; CEBPB, CCAAT-enhancer-binding protein-β; MODY, maturity-onset diabetes of the young; HIF-1α, hypoxia-inducible fator-1α; HTS, high-throughput screening; MSC, multicellular spheroid; UPR, unfolded protein response; BRB, blood retinal barrier; AAV, adeno-associated virus; EV, extracellular vesicle.

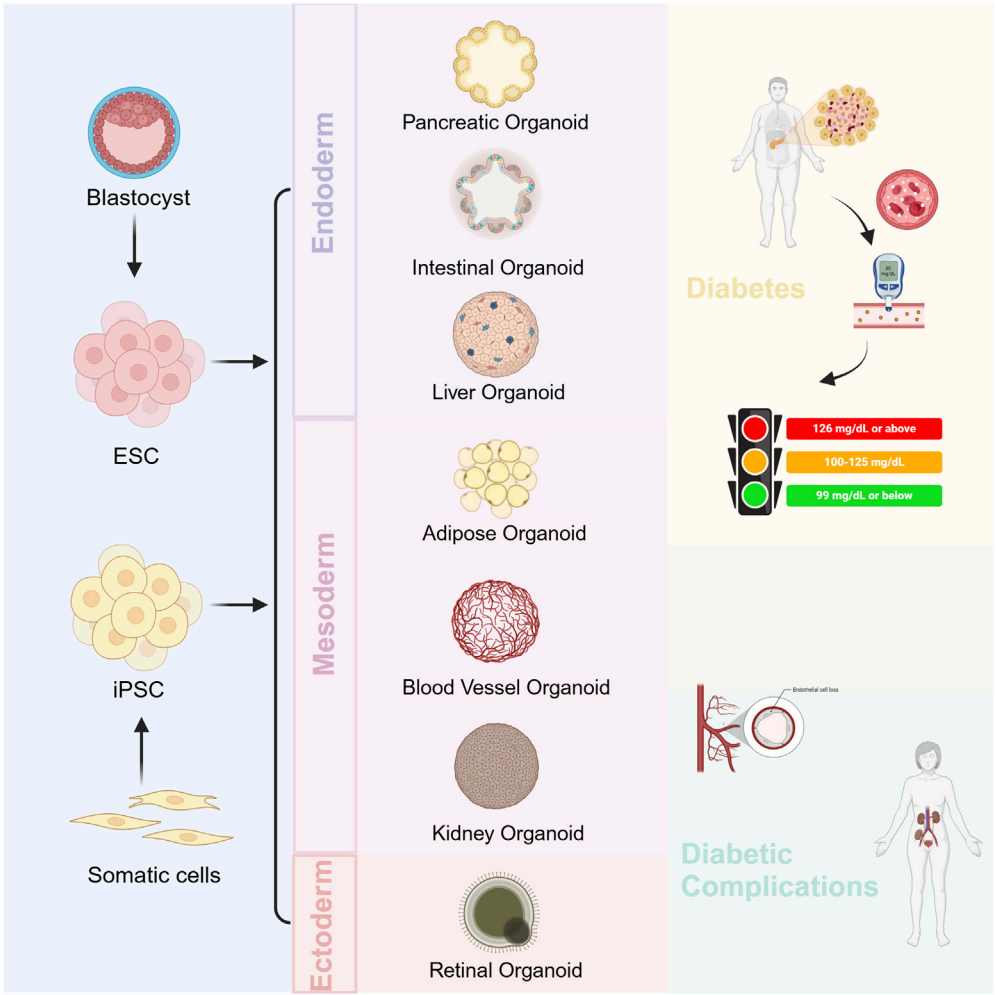

FIGURE 1
Organoids for the study of diabetes mellitus and its complications.

summarized the development of pancreatic organoids in terms of origin, establishment, optimization, and maturation (Figure 2).

2.1.1 Origin and establishment of organoids

Pancreatic organoids arise primarily from two species: mice and humans. Dissociated cell cultures from embryonic, fetal, or adult mouse pancreas can develop to form pancreatic organoids (Grapin-Botton and Kim, 2022). Human fetal cells, adult pancreatic tissue, and PSCs are also gradually being used to construct 3D organ tissues (Loomans et al., 2018; Grapin-Botton and Kim, 2022). With the gradual understanding of the mechanisms of pancreatic islet development, it was realized that cell fate could be designated by the pairing of various combinations of growth factors and small molecules (Zhang et al., 2022). Pagliuca et al. first generated high levels of NKX6.1+/PDX1+ co-expressing pancreatic progenitor cell clusters with the FGF family member KGF, the hedgehog inhibitor SANT1, and retinoic acid. Then attempted combinations of multiple signaling factors including wnt, activin, hedgehog, epidermal growth factor (EGF), transforming growth factor β (TGFβ), thyroid hormones, retinoic acid, and gamma secretase inhibition. Ultimately, human pluripotent stem cells (hPSCs) were successfully utilized to generate hundreds of millions of glucose-responsive β-cells, which were similar to primary β-cells and had an ultrastructure similar to that of adult β-cells (Pagliuca et al., 2014). Similar combinations and selection schemes have been shown in other reports of induction of functional β-cells as well (Nostro et al., 2015; Russ et al., 2015). Notably, the generation of ductal, acinar, and endocrine cells is inextricably linked to NKX6-1 expression, which is an important turning point in the differentiation of hPSCs into pancreatic β-cells. It has also been reported that the transcriptional repressor REST is an important endocrine regulator during pancreatic development and can affect the expression of endocrine genes or the formation of endocrine cells (Rovira et al., 2021). However, the addition of various induction factors should be appropriate, and may be influenced by the cell type selected. For example, there may be a dual-sided or cell-specific role for noggin in the culture of pancreatic islet-like organoids, where, on the one hand, it promotes the differentiation of progenitor cells but, at the same time, it may affect the expression of NKX6.1(Russ et al., 2015; Wesolowska-Andersen et al., 2020).

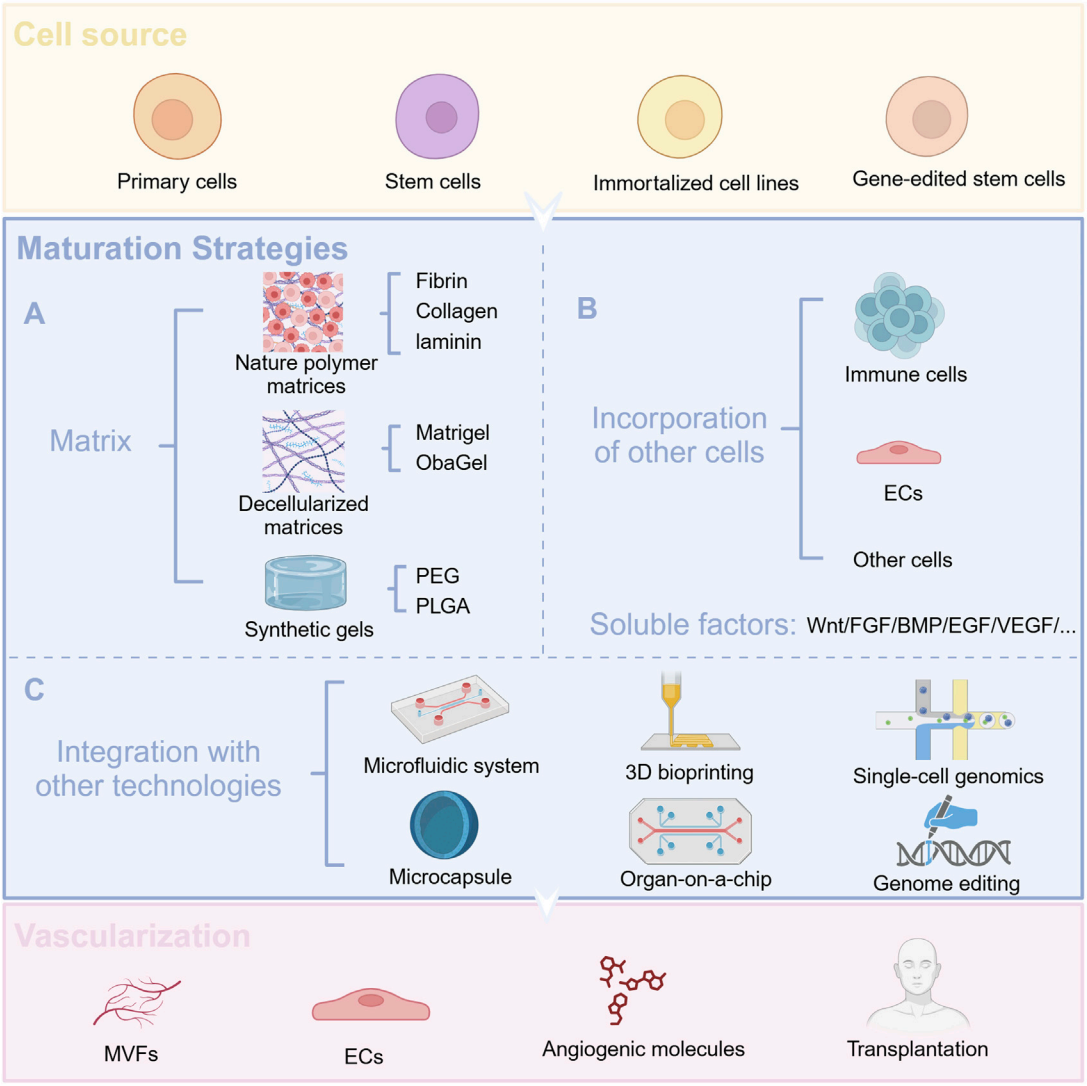

FIGURE 2
Cellular sources of organoids and optimization strategies. **(A)** Use different matrix gels to promote organoid maturation **(B)** Co-culture with other cells and add appropriate soluble factors to facilitate organoid maturation **(C)** Combine organoid technology with other advanced technologies.

Vitamin C has a similar role: on the one hand, it seems to promote sc-β cell maturation by inhibiting the premature expression of NGN3; however, the cells induced thereby lack glucose responsiveness (Rezania et al., 2014). Hence, we found that the evaluation of pancreatic islet organoids tends to focus on the expression of INS, GLUT2, MAFA, and NKX6.1/PDX1, as well as β-cellular hormone-specific INS genes, expression of C-peptide proteins, glucose-stimulated insulin secretion (GSIS), and higher calcium ion (Ca2+) fluxes.

2.1.2 Maturation strategies for organoids

In addition to being related to the cells used, induction time, cell density, inducer species and ratios, some dynamic culture modes, such as perfused 3D culture conditions, also promote organoid maturation and exhibit enhanced expression of PDX1 and NKX6.1. For example, Tingting Tao et al. used an organ-on-a-chip platform to create a human islet organoid microsystem containing a microfluidic device, which exhibited superior islet organoid differentiation and maturation compared with static culture (Tao et al., 2019). A number of microphysiological systems have also been developed, and studies have shown that such dynamic cultures are more suitable for organoid cultures than static ones (Patel et al., 2021). Furthermore, Carla A Gonçalves found that hPSC-derived pancreatic progenitor cells cultured in 3D were transcriptionally closer to the fetal pancreas, were not dependent on EGF supplementation and had the ability to expand and differentiate (Gonçalves et al., 2021).

The option of biomaterials is an essential step in driving the discovery of organoid technology and the application of regenerative medicine (Noro et al., 2024). Moreover, breakthroughs in materials technology and their integration with stem cell technology will also drive the maturation of organoid technology and contribute to more sophisticated experimental research and broader clinical applications (Liu et al., 2019). Matrigel, derived from Engelbreth-

Holm-Swarm mouse sarcoma cells, is a common culture for organoids; however, there are problems with batch variation and safety, and the composition and proportion of Matrigel cannot be accurately determined, which limits the utility of organoids in drug development and regenerative medicine. In view of this, several Matrigel-free culture methods have also been developed, including decellularized ECM, synthetic hydrogels, and gel formation of recombinant proteins (Kozlowski et al., 2021). Hydrogels with high water content have the outstanding advantage of high biocompatibility and can mimic the microenvironment of the natural extracellular matrix (ECM) by modulating their biochemical and physical properties to guide a range of cellular behaviors including cell adhesion, proliferation, migration, differentiation, and cell-cell/cell-matrix interactions in a 3D model (Liu et al., 2019). Hydrogels are further classified into natural and synthetic hydrogel. Natural hydrogels contain bioactive matrices with abundant cell adhesion sites and bionic scaffolds with the ability to encapsulate cells *in situ*, which is favorable for the reconstruction of organoid models *in vitro* (Liu et al., 2019). In contrast, synthetic hydrogels can be individually tailored in terms of composition and mechanical properties to enable more stable and excellent conditions for organoid formation, promote high reproducibility and organoid maturity, and can facilitate the formation of specific tissue/organ models (Liu et al., 2019). Joseph Candiello et al. utilized pancreatic progenitor cells derived from human ES cells and a novel hydrogel system, Amikagel, to generate regenerated islet organoids exhibiting cellular heterogeneity. Their findings demonstrated that Amikagel-induced globules exhibited expression of Pdx-1, NKX6.1, and INS1 genes and augmented C-peptide protein expression (Candiello et al., 2018). Deliang Zhu et al. also found that collagen VI is a key component for normal islet development, and their invention of a Col-VI-based biomimetic ECM can optimize the cell composition and endocrine function of islet organoids (Zhu et al., 2025).

2.1.3 Strategies for vascularization of organoids

Vascularization of islets in clinical transplantation therapy is also a pressing issue. The co-culture of cell lines, natural tissue fragments, and iPSC spheroids with human umbilical vein endothelial cells (HUVECs) and mesenchymal stem cells (MSC) in matrix gel has been used as one of the strategies to vascularize organoids. Human and mouse pancreatic islets with endothelial cells were able to self-organize in a spatiotemporal manner, and such vascularized islets were more effective in treating diabetic mice after transplantation (Takahashi et al., 2018). A polyethylene glycol-maleimide hydrogel and a protein hydrolysis degradable synthetic hydrogel have also been developed to enhance angiogenesis in transplanted pancreatic islets in a diabetic mouse model (Phelps et al., 2013; Weaver et al., 2017; Weaver et al., 2018). Hydrogel infusion showed significantly higher graft survival and superior glucose regulation properties and intra-islet angiogenesis compared with intravenous infusion of islets (Phelps et al., 2013; Weaver et al., 2017; Weaver et al., 2018). Additionally, a 3D printing technique applying a new tissue-specific bioink developed from pancreatic ECM and hyaluronic acid methacrylate has been applied in promoting the formation of pancreatic organoids and their vascular networks, which is expected to improve the effectiveness of islet transplantation (Wang et al., 2023).

2.2 Retinal organoids (ROs)

Retinal disease is a leading cause of visual loss and blindness and is associated with complex pathogenesis such as angiogenesis, inflammation, immune regulation, fiber proliferation, and neurodegeneration (Zhang et al., 2021). DR is one of the common microvascular complications of diabetes mellitus. It can cause severe vascular damage and neuronal impairment of the retina, and even result in loss of vision, which has a huge impact on patients' lives (Cheung et al., 2010; Antonetti et al., 2021). The retina is a highly complex vascularized tissue containing at least 60 functionally distinct cell types, in which various resident cell types communicate with each other and with cells from the blood and immune system (Chichagova et al., 2019; Zhang et al., 2021). Currently, it is also feasible to generate ROs. ROs exhibit consistency with *in vivo* retinogenesis and retinal morphology and can contain most retinal and neuronal cell types, including optic rod and cone cells, ganglion cells, bipolar cells, horizontal cells, amacrine cells, and Müller cells (Chichagova et al., 2019). ROs have been successfully established in many cases, and usually, the addition of appropriate exogenous factors such as BMP, Wnt, Nodal and Notch pathway inhibitors, some growth factors including insulin-like growth factor 1 (IGF1), bFGF, activin, SHH, and triiodothyronine (T3), and serum can induce cell differentiation and determine retinal progenitor cell (RPC) fate (Chichagova et al., 2019). Researchers have found that Dkk1 and Noggin play an important role in enhancing the differentiation of hESCs and hiPSCs to retinal progenitors and photoreceptor precursors at the early stages of differentiation (Mellough et al., 2012). Moreover, ES cells treated with SFEB, Dkk1, LeftyA, serum, and activin produce neural retinal precursors with the ability to differentiate photoreceptors (Ikeda et al., 2005). IGF1 signaling with retinoic acid and T3 is also important for retinal development (Chichagova et al., 2019). Moreover, researchers found that the addition of 9-cis retinoid acid, rather than the commonly used all-trans retinoic acid, accelerated the differentiation of optic rod photoreceptors in organoid cultures (Kaya et al., 2019).

Concurrently, simple and effective strategies have been created that do not require the addition of extrinsic signaling modulators or the involvement of natural retinal cells (Osakada et al., 2008; Zhong et al., 2014). For example, a two-step xeno-free/feeder-free culture system was developed to enable simple and efficient differentiation of hiPSCs into retinal cells (Reichman et al., 2017). In less than 1 month, walled hiPSCs were able to generate self-forming neural retina-like structures containing RPCs (Reichman et al., 2017).

2.3 Kidney organoids

hPSCs can differentiate into pluripotent renal unit progenitor cells that form renal unit-like structures and further differentiate into kidney organoids that can mimic kidney development and injury *in vitro* (Morizane et al., 2015). Moreover, 3D kidney organoids have also been successfully generated from different types of source cells, including adult/fetal kidney tissue and kidney cancer biopsies (Liu et al., 2022). In addition to typical kidney organoids, primary renal tubular epithelial organoids, called tubuloids, can be derived from human kidneys and urine and are

capable of mimicking key features of renal units in health and disease states (Schutgens et al., 2019; Stower, 2019). These kidney organoids exhibit basic functions such as tubular reabsorption represented by proximal tubular epithelium-mediated dextran uptake and functional renin secretion (Shankar et al., 2021). Xia Y et al. established a diabetes-like renal organoid using alternating 5 mM and 25 mM glucose every 24 h to mimic glucose level shocks in patients with diabetes and found that diabetic-like renal organoids and patients with diabetes had higher angiotensin-converting enzyme 2 (ACE2) expression levels (Xia et al., 2022). However, there are limitations to the application of renal organoids, for example, currently available protocols do not simulate fully mature kidneys; hence, replication of late-onset diseases using organoid technology is not convincing compared with early-onset diseases. It is also worth mentioning that the potential teratogenicity of organoids that are exposed to high-glucose environments should not be overlooked; therefore, a more robust, complete, and realistic renal vasculature system is urgently required (Nishinakamura, 2019; Woolf, 2019). These limitations are expected to be broken one by one with technological breakthroughs. The formation of vascularized glomeruli can be observed by transplantation of iPS-derived renal unit progenitor cells and concomitant transplantation of mixed aggregates of HUVECs and MSCs, among other modalities (Sharmin et al., 2016). In addition, a dynamic culture device, under *in vitro* flow, can induce renal organoid vascularization and promote further maturation of organoid morphology (Homan et al., 2019). Changing the medium composition ratio, by dynamically adjusting Wnt signaling at different stages of organoid differentiation, can produce vascularized organoids and a thickening of the basement membrane of the distal microvascular network, a phenomenon that is inextricably linked to many microvascular lesion (Low et al., 2019). Moreover, kidney organoids show some angiogenic capacity by intrathecal transplantation in chick embryos or by transplantation in chick embryo chorionic allantoic membranes, and organoid-derived endothelial cells can expand to form perfused capillaries and form a vascular network with host-derived blood vessels (Garreta et al., 2019; Koning et al., 2022).

Differentiation in a fully controlled and physiologically relevant 3D growth environment is essential to improve reproducibility and maturation of organoids. Suitable soft environments can accelerate the differentiation of hPSC-derived kidney organoids - the simulation of the intravitreal chorionic villous allantoic membrane microenvironment *in vitro* using a compliant hydrogel promotes the efficient generation of renal vesicles and unit structures (Garreta et al., 2019). Futhermore, after organoids were cultured in a fully synthetic peptide hydrogel, single-cell RNA sequencing showed that this culture mode could produce more mature organoids with fewer off-target cells (Treacy et al., 2023). In conclusion, the application of organoid technology to the study of DN has become possible; however, technological advances are still urgently neede.

2.4 Intestinal organoids

The intestine is an important organ involved in human metabolism. Either adult or embryonic stem cells can be used as a source of intestinal stem cells, either by obtaining them from crypt-containing stem cells or isolating individual Lgr5-expressing ISCs from human or mouse small intestinal or colonic tissues, or by direct differentiation of ESCs or iPSCs into 3D intestinal organoids (Zietek et al., 2015; Rahmani et al., 2019). Major components required for culturing intestinal organoids include Wnt-3a (W), EGF (E), Noggin (N), and Rspondin 1 (R) (Rahmani et al., 2019). Intestinal nutrient transport and sensing are also important in diabetes research, and mouse small intestinal organoids enable the study of nutrient and drug transport, sensing and secretion of enteric insulinotropic hormone, and intracellular signaling processes (Zietek et al., 2015). More importantly, glucagon-like peptide-1 (GLP-1), which is released by enteroendocrine cells in the intestine, plays an important role in insulin secretion, food intake, and intestinal peristalsis by increasing insulin secretion and inhibiting glucagon release, as well as delaying gastric emptying and suppressing appetite (Meier, 2012; Gribble and Reimann, 2021; Gribble and Reimann, 2021). Targeting GLP-1 is now an effective treatment for diabetes. Human ileal organoids designed by CRISPR-Cas9 technology have the ability to label and maintain human L cells, opening avenues for the development of drugs targeting the human enteroendocrine system (Goldspink et al., 2020). An intestinal-pancreatic cell co-culture model with 3D morphology also holds promise for screening GLP-1 analogs and stimulants for the treatment of diabetes (Nguyen et al., 2017). Another aspect of intestinal organoids that can be used for diabetes research and treatment is reflected in their cellular transformation capacity. Intestinal endocrine progenitor cells from mice have the potential to be converted into insulin-secreting cells by inhibiting Foxo one or stimulating the expression of Pdx1, MafA, and Ngn3, and can be used as a complementary source in diabetes transplantation therapy (Bouchi et al., 2014; Chen et al., 2014).

The structure and composition of hydrogels for cultured organoids can also be further optimized to promote the differentiation of various enterocyte cell types (Mulero-Russe and García, 2024). ECM hydrogel of gastrointestinal origin has been showen to be a suitable alternative to matrix gel in gastrointestinal organoid cultures, which mimics the organoid microenvironment *in vivo* and enables long-term passaging culture and transplantation of organoids (Kim et al., 2022). Additionally, hydrogel matrix hardness also regulates the trajectory of organoid differentiation (He et al., 2023). Moreover, the combination of intestinal organoids with micro-engineered microarray technology can further promote intestinal organoid maturation (Workman et al., 2018). In conclusion, intestinal organoids also provide a viable platform for the study of metabolic diseases such as diabetes mellitus.

2.5 Adipose organoids

Adipose tissue is a major site of insulin resistance in patients with type 2 diabetes mellitus (T2DM), and brown adipocytes (BAs) have also been recognized as a potential cell source for the treatment of metabolic diseases such as diabetes (Zhang et al., 2020; Hu and Lazar, 2022). Previously, researchers used hPSCs and added specific transcription factors-such as nuclear receptor peroxisome proliferator activated receptor γ (PPARγ), CCAAT-enhancer-binding protein-β (CEBPB), and PR domain containing 16 (PRDM16)-to induce the production of white and brown adipocytes (Ahfeldt et al., 2012). Transplantation of hiPSC-

derived adipocytes into mice produces well-vascularized adipose tissue and shows glucose uptake capacity (Guénantin et al., 2017), and transplanted BAs also reduce circulating blood glucose levels in hyperglycemic animals (Zhang et al., 2020). Adipose organoids are mainly formed by self-organization of adipose progenitor cells or hPSC and have a structure and function similar to that of adipose tissue (Beydag-Tasöz et al., 2023). Matrix components such as collagen, hydrogel, and ELP-PEI contribute to organoid formation (Hu and Lazar, 2022). Several scaffold-free methods have also been developed to produce 3D fat spheres that can achieve higher levels of lipocalin (Klingelhutz et al., 2018). Cell-to-cell and cell-to-environment interactions are also gradually being emphasized and modeled in adipose organoids (Taylor et al., 2020). In particular, researchers have developed a number of methods for vascularizing adipose organoids. For example, Muller et al. co-cultured human adipose-derived stem cells (hADSCs) with endothelial cells to form patient-specific vascularized adipose organoids that can secrete leptin and can be connected to the vascular system of host mice after transplantation (Muller et al., 2019). Alternatively, the use of adipose tissue-derived stromal vascular fraction or the adoption of other modalities has been shown to be an adipose organ vascularization strategy (Peirsman et al., 2023; Robledo et al., 2023). The use of the human stromal vascular fraction of white adipose tissue as a source of adipose and endothelial progenitor cells to generate vascularized and functional human beige adipose organoids has been reported in studies (Escudero et al., 2023). Retinoic acid also has the potential to promote the development of adipose blood vessels (Wang and Du, 2023).

2.6 Liver organoids

The liver primarily consists of epithelial, stromal, endothelial, and mesenchymal cells and is involved in body metabolism as well as exocrine and endocrine processes (Prior et al., 2019). Therefore, liver organoids can be used to study glucose metabolism and insulin resistance in the liver, as well as non-alcoholic fatty liver disease, which increases the risk of T2DM. Liver organoids have been established from iPSCs, ES cells, adult hepatocytes, and cells of adult tissue origin (Hu et al., 2018; Prior et al., 2019). Additionally, more and more liver organoids with self-renewal capacity and mature functions are being developed for disease modeling, drug screening, and precision medicine (Mun et al., 2019; Shinozawa et al., 2021). Moreover, technologies such as CRISPR-Cas9 can also contribute to this goal (Hendriks et al., 2021). A microfluidic multi-class organ system developed using a 3D co-culture of hiPSC-derived liver and pancreatic organoids has also been developed and exhibits activation of metabolism-related signaling pathways, and an increase in glucose utilization in the liver organoids (Tao et al., 2022). Furthermore, under hyperglycemic conditions, the organoid appeared dysfunctional and could be treated with metformin. Hence, this multi-organoid system can recapitulate the relevant liver-islet axis in humans under physiological and pathological conditions, and also provides a unique platform for future T2DM research and drug development (Tao et al., 2022). Moreover, multicellular human liver organoids consisting of hepatocytes, stellate cells, and Kupffer-like cells have been created, which can further mimic the complexity of real liver tissue (Ouchi et al., 2019). It is worth noting that the rational use of new materials has the effect of improving drug efficacy; however, their clinical application can be hindered by possible side effects such as liver toxicity. In this regard, liver organoids can also provide rapid toxicity screening of nanomaterials and contribute to the safe use of medicines (Zhang et al., 2024).

2.7 Other organoids

Diabetes can cause severe damage to the endothelial cells and pericytes of blood vessels, which in turn can lead to serious complications, including DR and DN. Wimmer et al. generated blood vessel organoids from hPSCs and modeled the influence of diabetes on the vasculature, such as basement membrane thickening. They used the organoids to identify DLL4 and NOTCH3 as risk factors for diabetic vasculopathy (Wimmer et al., 2019a; Wimmer et al., 2019b). Liu et al. summarized the methods of generating blood vessel organoids and organoid vascularization, with emphasis on the roles of Notch, Wnt, BMP, VEGF, and PDGF signaling pathways in vascular differentiation (Liu C. et al., 2020). Strategies for organ-specific diabetic vascular disease models have also been established, facilitating research into diabetic vascular complications (Naderi-Meshkin et al., 2023). Additionally, to mimic human muscle insulin resistance and to study the molecules responsible for it, an ihPSC-derived myotube was also invented which had significant defects in glucose metabolism (Iovino et al., 2016). Self-assembled cardiac organoids can also be used to study cardiac development and mimic maternal diabetes-induced congenital heart disease (Lewis-Israeli et al., 2021).

3 The role of organoids in diabetes research

Organoids can mimic the structure and function of organs *in vitro* and have a wide range of translational applications including disease modeling, drug screening, and cell therapy (Figure 3).

3.1 Organoids for pathogenesis studies

Organoids are widely-used in probing the pathogenesis and factors influencing diabetes mellitus. Through validation on null intestinal organoids, it was found that inhibition of intestinal Gpr17 expression could promote GLP-1 secretion and thus improve glucose metabolism, suggesting that GPR17 may be a potential interventional target for the treatment of diabetes mellitus (Yan et al., 2022). It was also discovered that miR-144, a micro-RNA involved in post-transcriptional regulation, could target IRG2 to regulate NRF1(Azzimato et al., 2021). miR-144 plays a key role in insulin resistance, and this result was also validated in a liver organoid: by silencing miR-144, the activity of fumarate hydratase, which plays a key role in the miR-144/IRG2/NRF1 pathway, was decreased (Azzimato et al., 2021). Using human brown fat organoids, Reverte-Salisa's research showed

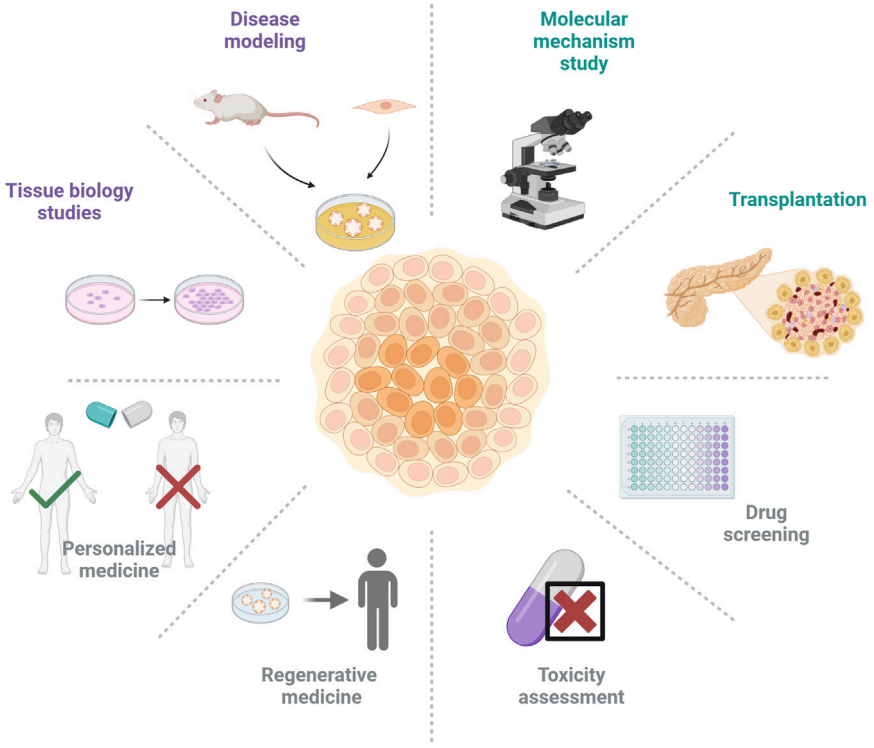

FIGURE 3
Various applications of organoid technology in the field of diabetes and its complications.

that EPAC1 can increase the proliferation and differentiation of brown fat cells, suggesting that EPAC1 may have the potential to increase energy expenditure and thereby combat metabolic diseases (Reverte-Salisa et al., 2024). Using liver organoids, L22RA1 was found to be closely related to liver lipid homeostasis, thereby affecting glucose tolerance and insulin resistance (Huang et al., 2025). Also, the development of human vascular organoids helps to gain a deeper understanding of the vascular effects caused by diabetes at the single-cell level and to identify diabetes-related genes (Nikolova et al., 2025).

Monogenic diabetes, including neonatal diabetes and maturity-onset diabetes of the young (MODY), is characterized by early onset. It is often caused by mutations in genes involved in pancreatic development and insulin synthesis and secretion (Aguilar-Bryan and Bryan, 2008; Bonnefond et al., 2023). However, people with T2DM can also suffer from monogenic diabetes (Bonnefond et al., 2023). Organoids are uniquely suited for research in this area, either by using patient-derived iPSCs and comparing them to CRISPR/Cas9 gene-corrected autologous cells, or by genetically modifying hPSCs to mimic the disease-related gene mutations of the patient. Based on this strategy, researchers identified and validated the roles of STAT3, NEUROG3, PDX1, ONECUT1, and MAFB in pancreatic and β-cell development and diabetes progression (Saarimäki-Vire et al., 2017; Wang et al., 2019; Russell et al., 2020; Philippi et al., 2021). They also demonstrated the mechanism of endocrine regulation by ONECUT1 in single-gene and multifactorial diabetes mellitus (Philippi et al., 2021). Endoplasmic reticulum stress is also present in these mutant phenotypes (Panova et al., 2022).

Organoids are also helpful in studying the effects of environmental factors and the body's metabolic state on tissues and organs *in vivo*. Using intestinal organoids, researchers have validated the powerful activation of peroxisome proliferator-activated receptor δ (PPAR-δ) by a high-fat diet (Beyaz et al., 2016). To ensure normal insulin secretion, pancreatic β-cells were exposed to glucose and oxygen for a long period of time. This β-cell exposure to high glucose concentrations *in vitro* also leads to a hypoxic phenotype and activation of hypoxia-inducible factor-1α (HIF-1α), which is inextricably linked to glucose metabolism, oxidative stress, and angiogenesis (Gunton, 2020; Catrina and Zheng, 2021; Li et al., 2021). Coherently, insulin secretion-related indices improved by applying the HIF-1α inhibitor PX-478 to human islet organoids exposed to hyperglycemia (Ilegems et al., 2022). Researchers have worked to keep pancreatic progenitor cells *in vitro* close to their counterparts *in vivo*; hence, the resulting pancreatic organoids can be more accurately tested for the effects of genes that predispose to diabetes or other diseases of the pancreas (Gonçalves et al., 2021). Moreover, as adipose tissue is highly heterogeneous with many cell types (Corvera, 2021), adipose organoids are more than capable of further simulating real adipose tissue. A type of adipose spheroid sensitive to environmental changes was created to provide a system for assessing the effects of different factors on adipocytes (Klingelhutz et al., 2018).

With a deeper understanding of the molecular regulatory mechanisms of a disease, organoids can be instrumental in precision medicine and personalized therapy. Thiazolidinediones target the transcriptional activity of PPARγ to increase insulin sensitivity and reverse insulin resistance in patients with T2DM. Wenxiang Hu et al. used human adipose stem cell-derived adipocytes to demonstrate that the specific single nucleotide polymorphism rs4743771 could regulate the PPARγ target gene ABCA1, and that single nucleotide polymorphisms were enriched in the patient-specific PPARγ binding site, which correlates with the individual-specific effect of the azolidinediones drug rosiglitazone, explaining the inefficacy of this type of drug in some patients with diabetes(Nanjan et al., 2018; Hu et al., 2019). This model for studying human genetic variation in response to antidiabetic drugs may have important implications for the development of personalized therapies for metabolic disorders.

3.2 Organoids for drug development and screening

Organoid technology also shows the potential for the high-throughput screening (HTS) of drugs. Using hESC-derived glucose-responsive cells, mutations in CDKAL1, KCNQ1, and KCNJ11 were determined to cause impaired glucose secretion. On the basis of this, a further screen identified a drug candidate that could rescue CDKAL1-specific defects by inhibiting the FOS/JUN pathway (Zeng et al., 2016). A drug candidate, Galunisertib, was found to rescue GLIS3 mutation-induced β-cell death using a chemical screen based on a derivative of GLIS3$^{-/-}$ hESCs (Amin et al., 2018). Functional stem cell-derived β-cells for type 1 diabetes were developed and the efficacy of the diabetes drugs toluenesulfonylurea, liraglutide, and GCK activators was validated in this model (Millman et al., 2016).

Pathological damage can be induced in hepatic organoids using oleic and palmitic acids, and these organoids can be used as a platform for drug screening to assess toxicity and efficacy. For example, the antidiabetic drug troglitazone, which was withdrawn from the market due to its severe idiopathic hepatotoxicity, showed toxicity in the organoids. Moreover, the effects of metformin could be evaluated in the organoids and showed favorable results (Mun et al., 2019).

Diabetes is a multi-organ systemic disease in which various organs and tissues interact with each other in the development of the disease. A microfluidic multi-organoid system that mimics the human liver-islet axis has been established, which allows co-culture of hiPSC-derived liver and islet organoids (Tao et al., 2022). In this organoid system, metabolism-related pathways are activated, and pancreatic islet organs can secrete insulin and further contribute to increased glucose utilization in liver organoids (Tao et al., 2022). Meanwhile, the pathological state was simulated using high glucose conditions, which showed pathological damage consistent with the body's response. Moreover, this damage could be alleviated by metformin suggesting that this multi-organoid system can effectively mimic the liver-islet axis in either physiological or pathological states and provide a feasible solution for disease research and drug development.

At present, diabetes treatment not only requires blood glucose control but also management of vascular diseases, kidney diseases, and obesity. As mentioned earlier, GLP-1 agonists play a significant role in diabetes treatment, offering both blood glucose control and vascular protection. Pinto utilized human intestinal organoids to assess the efficacy of the GLP-1 analog semaglutide. They optimized its formulation and found that nanoparticles targeting the intestinal Fc receptor enhanced the absorption of semaglutide (Pinto et al., 2024). Qi Lin and colleagues established a microphysiological system comprising hiPSC-derived white adipocytes, hepatocytes, and macrophages, and further discovered that semaglutide can improve hepatocyte function by targeting adipocytes (Qi et al., 2024). However, the needs of some obese diabetic patients remain unsatisfied when using GLP-1 agonists alone. Tirzepatide is a dual receptor agonist for the glucose-dependent insulinotropic polypeptide (GIP) receptor and the GLP-1 receptor. Clinical studies have demonstrated that tirzepatide has good safety profiles. Compared to semaglutide, it shows better blood sugar control and weight loss effects, significantly delaying the progression of diabetes (Garvey et al., 2023; France and Syed, 2024; Aronne et al., 2025; Jastreboff et al., 2025). E Lorza-Gil and colleagues established an organoid model of pancreatic adipose tissue, whose adipogenesis marker levels are comparable to those of natural pancreatic adipocytes. This model not only effectively simulates the pancreatic fat-islet crosstalk but also retains donor-specific metabolic memory (Lorza-Gil et al., 2025). Using this organoid model to evaluate the therapeutic effects of tirzepatide, the study demonstrated that tirzepatide stimulates lipolysis and reduces levels of inflammatory factors IL-6 and MCP-1 (Lorza-Gil et al., 2025). And I believe that the application of vascular organoids and vascularized organoids will also further evaluate and validate the vascular and cardiovascular protective effects of tirzepatide.

These examples suggest that drug candidates that rescue gene-specific defects can be identified using organoid technology, paving the way for precision therapy of metabolic diseases. We also summarized organoids used in diabetes mechanism research and drug screening (Table 1).

3.3 Organoids for organ transplantation and cellular replacement therapy

Cell therapy is considered a viable option in the treatment of diabetes, replenishing ß-cells and maintaining glucose homeostasis through pancreas or islet transplantation (Lysy et al., 2013). Clinically, a 68 kg person with type 1 diabetes needs about 340–750 million transplanted islet cells. Organoid technology allows for the suspension of 300 million cells in a single 500 mL flask, which undoubtedly alleviates the shortage of donors in the clinic (McCall and Shapiro, 2012; Pagliuca et al., 2014). Researchers found that introducing vitamin C at an early stage of pancreatic progenitor cell differentiation led to the generation of PDX1/NKX6.1 pancreatic progenitor cells, and that further differentiation using agents including ALK5 inhibitors, BMP receptor inhibitors, and thyroid hormone led to the upregulation of NGN3 and a population of cells a large fraction of which co-expressed PDX1, NKX6.1, NEUROD1, and NKX2.2(Rezania et al., 2014). Addition of notch inhibitors to the above leads to the

TABLE 1 Organoids for diabetes mechanism research and drug screening.

Source/Methods	Organoid types	Treatment	Main research target	Validated drugs	Refs
intestinal Gpr17 knockout mice	intestinal organoid	$Gpr17^{-/-}$	Gpr17	-	Yan et al. (2022)
co-cultures of hepatocytes and non-parenchymal cells	human liver organoids	miR-144 silencing	miR-144	-	Azzimato et al. (2021)
Patient-derived iPSCs	Differentiation of PSCs into β-like cells	-	ONECUT1	-	Philippi et al. (2021)
hiPSC	human brown fat organoids	preferential activator of EPAC1	EPAC1	-	Reverte-Salisa et al. (2024)
hiPSCs	human liver organoids	-	IL22RA1	-	Huang et al. (2025)
SVF-derived organoids	human pancreatic adipose tissue organoids	-	GLP1R/GIPR	Tirzepatide	Lorza-Gil et al. (2025)
HUES9 cells	human intestinal organoids	semaglutide was incorporated into PLGA-PEG nanoparticles	intestinal Fc receptor	Semaglutide	Pinto et al. (2024)
Patient-derived iPSCs	human islet organoids	high glucose	HIF-1α	HIF-1α inhibitor PX-478	Ilegems et al. (2022)
hESC	pancreatic organoids	$CDKAL1^{-/-}$	CDKAL1	T5224	Zeng et al. (2016)
hESC	mono-hormonal glucose-responding pancreatic β-like cells	$GLIS3^{-/-}$ hESC	GLIS3	Galunisertib	Amin et al. (2018)
Patient-derived hiPSCs	functional stem cell-derived β-cells	glucose	-	Sulfonylurea, liraglutide, GCK activators	Millman et al. (2016)
PSCs	human hepatic organoids	oleate and palmitate	-	Troglitazone, metformin	Mun et al. (2019)
hiPSC	Islet organoids, liver organoids	high glucose	-	metformin	Tao et al. (2022)

generation of cell populations in which a large proportion of PDX1/NKX6.1/NEUROD1 cells express insulin (Rezania et al., 2014). These highly differentiated cells rapidly reversed diabetes after transplantation in mice (Rezania et al., 2014). Moreover, patient-derived hiPSCs can differentiate to generate PDX1+/NKX6-1+ cells, which can be transplanted into mice to spontaneously generate glucose-responsive cells (Millman et al., 2016).

Furthermore, the ability to transform terminally differentiated cells could also be a powerful tool for treating diabetes. With the help of the reprogramming factors Pdx1, MafA, and Ngn3 (PMN), researchers were able to transform intestinal crypt cells into endocrine cells, which were shown to be glucose-responsive and able to ameliorate hyperglycemia in diabetic mice. Moreover, PMN has the ability to spur the transformation of intestinal epithelial cells into beta-like cells in human intestinal-like organoids (Chen et al., 2014). Recently, Eiji Yoshihara et al. added hADSCs and HUVECs during the differentiation of hiPSC-derived endocrine progenitor cells to form multicellular spheroids (MCSs) that were comparable to the size of human pancreatic islets and contained insulin-secreting cells. MCSs transplanted into the renal capsule were able to maintain glucose homeostasis in STZ-induced NOD-SCID mice for a period of time and could regulate insulin secretion according to the state of the organism, which was also similar to human insula transplantation (Yoshihara et al., 2020). In a study by Daisong Wang et al., a novel islet progenitor cell, Procr islet cell, was identified and cultured *in vitro* to become an islet-like organoid that included all endocrine cell types (Wang D. et al., 2020). The organoid was transplanted into the kidney capsule of STZ-induced diabetic mice and showed that the organoid could secrete insulin and attenuate weight loss and hyperglycemia in mice, showing similar effects to those with fresh islet transplantation (Wang D. et al., 2020). In addition, the integration of human amniotic epithelial cells has been reported to enhance the transplantation success of pancreatic islet organoids (Lebreton et al., 2019). Interestingly, it has also been suggested that intestinal cells can be transformed by cellular reprogramming into cells that can produce insulin (Du et al., 2022). Similarly, human gastric stem cells were utilized to differentiate into islet organoids containing gastric insulin-secreting cells, and such gastric-derived human insulin-secreting organoids could effectively restore glucose homeostasis in mice. It is worth mentioning that human gastrointestinal stem cells can be easily biopsied, and can be successfully cultured and expanded *in vitro*, providing a precious resource for autologous cell therapy (Huang et al., 2023). However, secretion of islets after transplantation is still an issue to be considered, which may be related to islet size (Fujita et al., 2011; Zhang et al., 2022).

Adipocytes derived from hPSCs also provide a unique program for studying metabolic diseases. BAs are considered a potential cell source for treating metabolic diseases such as diabetes (Zhang et al.,

2020) as their increased activity also contributes to insulin sensitivity in patients with diabetes (Sakers et al., 2022). Several teams have successfully induced adipocytes and found that PPARG2, CEBPB, and PRDM16 play important roles for the differentiation of mesenchymal progenitor cells derived from PSCs to white or brown adipocytes. When these induced adipocytes were transplanted into mice, they had mature morphological and functional characteristics (Ahfeldt et al., 2012). A protocol for generating BAs from hPSCs via a paraxial mesoderm intermediate shows that transplanted BAs exhibit multilocular lipid droplet morphology and elevated UCP1 expression, increased metabolism in recipient mice, reduced circulating glucose levels in hyperglycemic mice, and no tumorigenic effect (Zhang et al., 2020). It is also worth mentioning that engineered adipocytes created using CRISPR-Cas9 technology-which is best characterized by modifying white adipocytes to a BA phenotype-improved glucose tolerance and insulin sensitivity in mice after transplantation. This suggests that this means of modification may open up another unique therapeutic direction for obesity and diabetes (Wang C.-H. et al., 2020).

4 The role of organoids in diabetic complications

4.1 Diabetic retinopathy (DR)

First, it is feasible to utilize organoids for the study of pathological mechanisms of DR. In addition to the high degree of structural and functional similarity to the human retina, more importantly, the cell type specificity of disease-related gene expression present in the human retina is present in the organoid (Cowan et al., 2020). Furthermore, a study by Capowski et al. showed that ROs could be generated repeatedly from 16 hPSC lines, reducing inconsistencies between cell lines (Capowski et al., 2019). The three stages of retinal organoid growth can also be easily distinguished morphologically by optical coherence tomography (Capowski et al., 2019). Taken together, this makes it feasible to use organoids to study disease mechanisms and to undertake targeted repair of the human retina (Gagliardi et al., 2018; Miltner and Torre, 2019; Cowan et al., 2020). For example, researchers recently developed a physiologically similar 3D Outer Blood-Retinal Barrier model with choroidal capillaries (Nam et al., 2023). When simulating diabetic pathological conditions, the model shows typical features seen in patients with diabetes, such as reduced tight junctions and impaired barrier functions (Nam et al., 2023). Additionally, recent studies utilizing ROs have found that an unbalanced unfolded protein response (UPR) is associated with DR-related retinal toxicity caused by 1-deoxysphingolipids, and that ATF6 activation attenuates 1-dSL toxicity (Rosarda et al., 2023). This suggests that targeting 1-dSL and the UPR is promising for the treatment of DR (Rosarda et al., 2023). This also reveals the feasibility of utilizing ROs to study DR-related mechanisms.

Interestingly, hiPSC-derived ROs have been reported to release exosomes and microvesicles with small non-coding RNAs that have been implicated in the regulation of human retinal development (Zhou et al., 2021). Exosomes play a special and important role in many biological barriers, including the blood-brain barrier and blood retinal barrier (BRB), which may promote retinal development or accelerate the development of retinal diseases, including DR (Elliott and He, 2021). On the one hand, in DR, exosomes in platelet-rich plasma have been observed to cause retinal endothelial damage through upregulation of the TRL4 signaling pathway (Liu J. et al., 2020). On the other hand, exosomes from retinal astrocytes contain anti-angiogenic components that inhibit choroidal neovascularization, protecting the eye from angiogenesis and maintaining its functional integrity (Hajrasouliha et al., 2013).

Second, the role of ROs in drug screening and delivery can be reflected in the following aspects. Organ-on-a-chip technology combining iPSC-organoids, with the advancement of bioengineering technology and the introduction of microfluidic systems, can better mimic the *in vivo* microenvironment and facilitate long-term RO maintenance; consequently, becoming a good platform for drug development (Kutlehria and Singh, 2021; Xue et al., 2021). For example, patients with diabetes have higher expression of HIF and HIF-regulated vasoactive mediators. Using mouse models and ROs, researchers have demonstrated that the newly developed HIF inhibitor 32-134D shows promise for clinical development; that is, it can effectively inhibit HIF accumulation and regulate related gene expression, thereby preventing retinal neovascularization and increased vascular permeability, which are typical features of DR (Zhang et al., 2023). Moreover, intraocular injection of various drugs and retinal gene therapy using adeno-associated virus (AAV) delivery are effective and widely adopted therapies for the treatment of DR and many other ophthalmic diseases (Trapani and Auricchio, 2018; Yu et al., 2023). Therefore, the human retinal microarray model integrating iPSC-ROs and retinal pigment epithelial cells in a microfluidic platform has the potential to test the performance of different types of AAV vectors (Achberger et al., 2021). In addition, the human retinal microvessel chip can mimic the human BRB and thus can be used for drug discovery and to test the effect of drugs on the barrier properties of the BRB (Ragelle et al., 2020). Furthermore, extracellular vesicles (EVs) can not only load specific molecular cargoes, but also have strong targeting capabilities and the ability to cross certain biological barriers. hiPSC-derived ROs have been found to release EVs and are therefore emerging as a promising platform for drug delivery (Zhou et al., 2021). In conclusion, organoids provide a more reliable preclinical model that can be used for drug clinical translational research.

Finally, ROs have potential applications in organ transplantation and cellular replacement therapy. Given the interdependence of photoreceptors, retinal pigment epithelial cells, and the choroidal capillary complex, almost any advanced retinal disease process will eventually result in the loss of all these tissues (MacLaren et al., 2016). Therefore, cell replacement or regeneration may be required for patients who develop end-stage retinal degeneration. ROs derived from PSCs contain all major retina-specific cell types: anaplastic cells, bipolar cells, horizontal cells, retinal ganglion cells, Müllerian glial cells, rods, and cones, and even have a hierarchical structure (Jin et al., 2019; Afanasyeva et al., 2021). Thus, reliable ROs provide an adequate cell resource for transplantation. The Lancet reported the first successful transplantation of a stem cell source for human retinal disease, and the findings suggest that hESC-derived cells could provide a potentially safe new source of cells for the treatment of a variety of

medical diseases requiring tissue repair (Schwartz et al., 2015). Recently, naïve hiPSCs (N-hiPSCs) have also been used in the study of DR (Park et al., 2020). Naïve diabetic vascular progenitor cells differentiated from N-hiPSCs have excellent high epigenetic plasticity and genetic stability, and can effectively migrate to damaged retinal sites, providing a viable direction for regenerative medicine (Park et al., 2020).

4.2 Diabetic nephropathy (DN)

Organoid technology has paved the way for the study of DN. As we all know, genetic factors may be one of the important determinants of the incidence and severity of DN, and hereditary lesions of glomerular structures may cause proteinuria (Liu et al., 2022). Using patient-derived kidney organoids, researchers found that mutations in the NPHS1 gene can cause congenital nephrotic syndrome (Tanigawa et al., 2018). Jun Wei Chan et al. verified the role of the HNF1A/SLC51B/estrone sulfate pathway in renal development and diabetogenesis using hiPSC-derived renal organoids from patients with MODY (Chan et al., 2023). Diabetic renal fibrosis is the basic pathological feature of DN. The degree of fibrosis is an important marker of the progression of DN, from diffuse thickening of the glomerular basement membrane in the early stage to glomerulosclerosis or tubular atrophy in the late stage (Ibrahim and Hostetter, 1997; Bloomgarden, 2005; Xiang et al., 2020). Xiaoping Yang and colleagues found that bile acid receptor agonists could reverse TGF-β1-induced renal fibrosis by regulating the Farnesoid X receptor, p-SMAD3, and TAZ in iPSC-derived renal organoids, demonstrating that bile acid receptor agonism exists in the early stage of renal fibrosis (Yang et al., 2024). Elsewhere, organoids could also shed new light on how diabetes leads to renal susceptibility to COVID-19 (Xia et al., 2022). The renin-angiotensin-aldosterone system plays an important role in blood pressure regulation, and the action of angiotensin II on the AT one receptor promotes vasoconstriction, which in turn has additional effects on renal blood flow and glomerular capillary pressure (Bloomgarden, 2005). The use of renal organoids has revealed that ACE2 increases the susceptibility of patients with diabetes to COVID-19 infection (Garreta et al., 2022).

Regarding drug screening and toxicity testing, organoids are potentially powerful tools for HTS. A fully automated HTS-compatible organoid platform has been developed for drug safety and efficacy prediction (Czerniecki et al., 2018). The kidney is an important metabolic organ of the body and kidney organoids have potential for toxicity testing and assessment (Fritsche et al., 2021). Moreover, experts suggest that new therapies currently in clinical trials for chronic kidney disease, including dual angiotensin receptor/endothelin receptor blockers and sodium-glucose cotransporter two inhibitors, can also be designed and optimized using organoids (Ramos et al., 2020).

5 Conclusion and future perspectives

Organoids are derived from ESCs and iPSCs from healthy individuals or patients, as well as organ-restricted ASCs, which can be cultured *in vitro* to build 3D structures and mimic the cellular heterogeneity, structure, and function of human organs. In recent decades, researchers have successfully generated various types of organoids and utilized organoid technology for disease modeling and drug screening. More importantly, Organoids have been found to have great clinical therapeutic potential. Consequently, we reviewed the progress of organoid application in diabetes and its complications, and demonstrated the broad potential of organoid technology in disease research, aiming to provide a theoretical basis and methodology for the study of related diseases.

Currently, there are several common strategies for modeling various diseases using organoid technologies. One involves the use of patient-derived hiPSCs and CRISPR-CAS technology for modeling various genetic diseases and exploring the role of a specific gene in disease progression. Another involves modelling environmentally induced acquired pathologies by exposing organoids to media supplemented with specific inducing factors or by simulating disease-inducing environments (e.g., hyperglycemic or hypoxic environments). Moreover, it is also possible to explore the driving factors of various pathologies in the human body and to screen for drivers that either ameliorate or exacerbate the phenotype of a disease (Beydag-Tasöz et al., 2023).

Although organoid technology with its unique innovation and creativity is rapidly evolving, and many types of organoids have promising applications in the field of research on diabetes and its complications, there are still several obstacles that need to be addressed. Therefore, we also summarized the current limitations and challenges of organoids technology (Figure 4), which may help indicate a feasible direction for better development of organoids.

First, there is still a gap between organ tissues and natural organs, which does not faithfully reflect the cellular diversity of the original tissues or the crosstalk and influence between different organs in an organism. Hence, reproducibility may be poor. Moreover, high intrinsic variability may mask therapeutic effects when validating drug action (Rossi et al., 2018; Bock et al., 2021). For example, when studying diseases, immune and inflammatory factors play a significant role, whereas organoid tissues often do not contain immune cell types (Richards et al., 2020).

Second, some organoid models tend to more closely resemble the state of the organ at the fetal stage (Shinnawi et al., 2019); hence, they can only simulate early-onset disease or the early stages of disease. During organoid culture, organoids lacking a vascular system may cause apoptosis or even tissue cavities due to hypoxia and nutrient deficiency (Fantin et al., 2013; Giandomenico et al., 2019). There have been some efforts to overcome these obstacles, including the use of small molecule compounds (e.g., BDNF) to precondition the organoids, which can develop over a longer period (Quadrato et al., 2017). Moreover, the teratogenicity that may be introduced when modeling the environment in which the disease occurs should be considered. Investigations are need as to whether this interferes with the screening of disease targets and shortens the lifespan of the organoid, which is itself one of the limitations of organoids.

To make up for these deficiencies, many vascularization strategies have been developed, which can well prolong the life span of organoids, promote tissue maturation, and simulate the microenvironment of the organoids so as to make them closer to the real situation. Currently, the commonly used vascularization

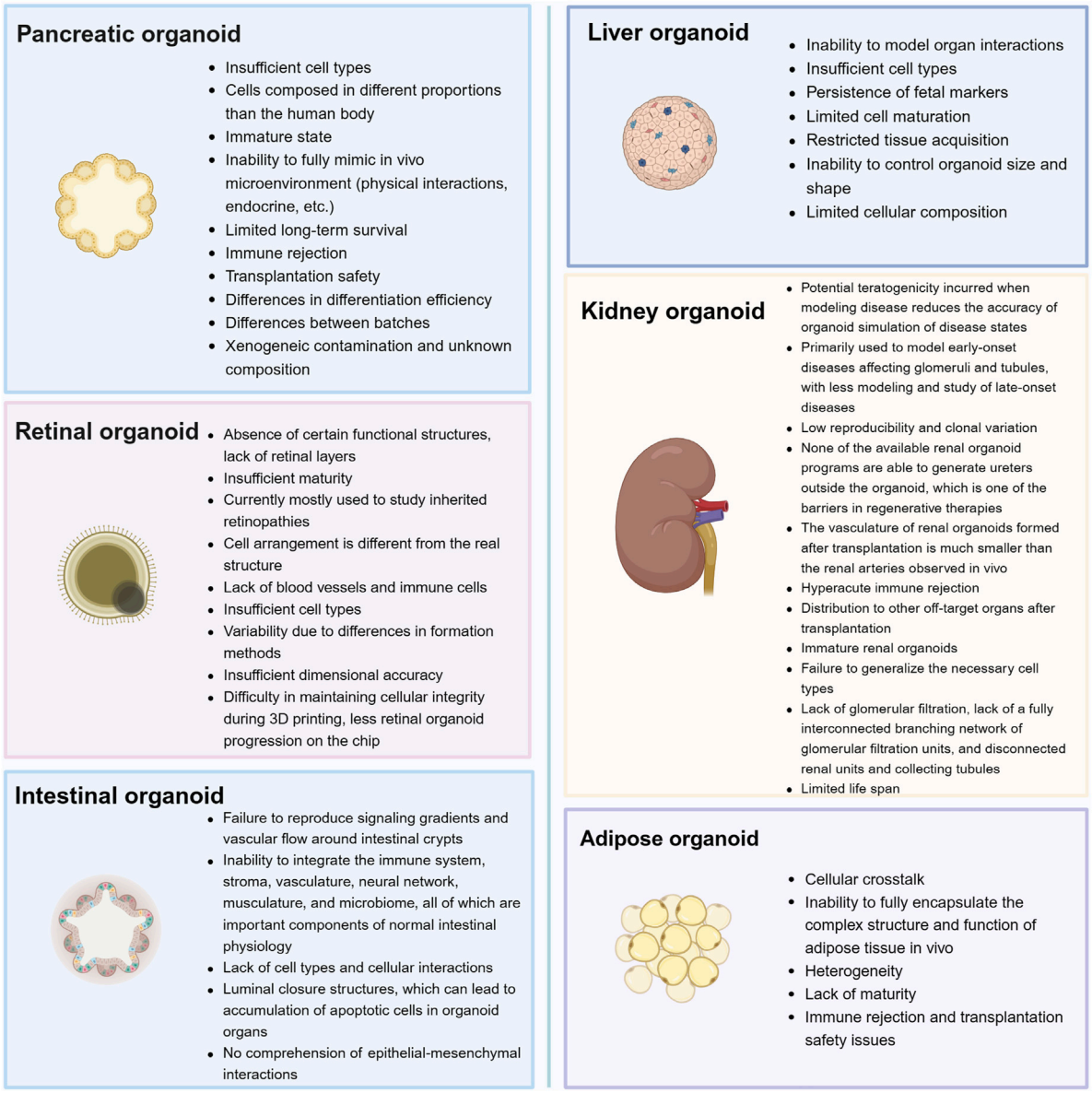

FIGURE 4
Current challenges and perspectives for organoids that can be used in the study of diabetes and its complications.

strategies are also divided into two types: *in vitro* and *in vivo*. *In vitro* methods include co-culturing organoids with endothelial cells, while *in vivo* methods mainly involve transplanting organoids into immunodeficient mice. Various studies have shown that transplanted organoids can be effectively vascularized and connected to the host mouse circulatory system. It is worth mentioning that more vascularization methods, such as microfluidic devices, have been developed by combining them with bioengineering and nanotechnology (Matsui et al., 2021).

There are many more modeling methods to think about and improve, and more organoid models need to be created. Some of the organoid models presented in this review are mostly used to simulate spontaneous diseases. This begs the questions: how can we better simulate acquired diseases? Can glomerular filtration limitation be realized using bioengineering techniques? It is believed that with further development of assembly and vascularization techniques, organoids will gradually mature and improve. In a nutshell, the 3D organoid system greatly complements the existing modeling system and may play a significant role in future basic and clinical research.

Author contributions

XX: Writing – review and editing, Writing – original draft. YZ: Writing – review and editing. YG: Writing – review and editing. YL: Supervision, Conceptualization, Writing – review and editing, Funding acquisition. XS: Writing – review and editing.

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Conflict of interest

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