



Technical advances in pluripotent stem cell-derived and tumorigenic organoids

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Cell culture systems have been widely used to address fundamental questions in biology without sacrificing animals. Three-dimensional (3D) organoids provide more information on *in vivo* conditions than traditional culture systems because multiple cell types in organoids interact with each other in 3D structures. Despite extensive research and advances in the organoid field, some important limitations remain and need further consideration. In this review, we summarize how organoids are generated from pluripotent stem cells and describe the recent technical progress that has made organoids more similar to *in vivo* tissues for the application of organoids to modeling cancer. Lastly, we briefly discuss some limitations that have been raised in this field.

Keywords: Organoids; Stem cells; Tumor; Spheroid; Assembloid

Introduction

Historically, the term “organoid” was first used in a tumor case study to describe certain histological features [1]. Organoids generally refer to tissues or structures that mimic an organ *in vitro*. After the establishment of a murine intestinal organoid derived from adult stem cells (ASCs), many types of organoids have been generated from various tissues [2]. Compared with conventional 2-dimensional (2D) culture systems, 3-dimensional (3D) organoids in polymeric matrices like Matrigel show higher similarity to organs in terms of gene expression and tissue architecture. Over time, technologies have been developed for generating organoids from many sources, such as tissue-resident ASCs, biopsy tissue specimens, or pluripotent stem cells (PSCs). Currently, organoids are regarded as effective model systems and have been widely adopted in recent studies to understand the stem cell niche and the tumor microenvironment (TME).

In an ASC-derived organoid model system, single stem cells or clusters of cells are embedded into an extracellular matrix that enables them to grow in a 3D stem cell niche or tissue-like structure [3]. Thus, organoids established in a 3D culture system mimic the structure and function of the corresponding organs. For example, intestinal organoids not only consist of various cell types such as goblet, entero-endocrine, absorptive, and Paneth cells, but also produce mucus and absorb and secrete biomolecules [4]. However, the main limitation of ASC-derived organoids is the absence of tissue-resident stem cells in some organs. In this aspect, PSC-derived organoids are promising platforms for modeling organs that cannot be easily obtained from tissue specimens. Unlike ASC-derived organoids, PSC-derived organoids recapitulate the differentiation of the developing embryo into specific organ types and contain various cell populations that closely mimic the tissue.

Organoid model systems serve as alternative organs or tissues

to study development and diseases, although some limitations still need to be overcome, especially the inability of any established organoid model systems to reproduce the entire functional repertoire of the corresponding organ, the limited lifespan of organoids, and the inefficiency of organoid formation. Nonetheless, many efforts have been made to establish various organoids for numerous organ types because organoids have great potential. Here, we briefly describe the methods and recent advances in the establishment and usage of organoids and propose future directions for refining this technology and expanding its applications.

Ethics statement: This study was a literature review of previously published studies and was therefore exempt from institutional review board approval.

Pluripotent stem cell-derived organoids to mimic *in vivo* tissues

During embryonic development, 3 germ layers are formed through the differentiation of PSCs. Here, the representative organoids from each germ layer are introduced, along with relevant technical advances. The techniques and characteristics of each organoid described here are listed in Table 1 [5–51].

1. Ectodermal organoids

1) Brain

The neuro-epithelium is induced in self-organizing PSCs cultured in serum-free media, and evaginates to form the neural retina, but timed notch signaling generates photoreceptors.

Table 1. Methodology and recent advances in organoid model technology

Germ layer	Organ	Methods	Advances	Reference
Ectoderm	Brain	Serum-free, minimal media, Embedded in Matrigel for structural support	Region-specific organoids Assembloids Vascularization Co-culture (microglia, astrocytes)	[5–22]
	Retina	Serum-free, minimal media+Wnt inhibitors in dissolved Matrigel All-trans-retinoic acid treatment promotes maturation	Extremely long-term culture (~150 days)	[23–26]
	Intestine	Adult stem cells: intestinal medium (N2, B27)+EGF+Noggin+R-spondin (ENR medium) Pluripotent stem cells: Activin-A → ENR medium Embedded in Matrigel	Implantation rescued macular degeneration Self-developing optic cups in cerebral organoids Organoids containing functional ENS Mesenchyme-free organoids	[27–30]
	Colon	Adult stem cells: intestinal medium + Wnt3a Pluripotent stem cells: BMP activation(mid/hindgut) → intestinal medium+EGF Embedded in Matrigel		[31–35]
Endoderm	Stomach	Adult stem cells: ENR medium+gastrin+FGF10+Wnt3a+N-acetylcysteine Pluripotent stem cells Antrum: intestinal medium+EGF+Noggin+Retinoic acid Fundus: intestinal medium+Wnt activation+FGF10 Embedded in Matrigel	Gastric assembloid (contains mesenchyme, smooth muscle, ENS)	[36–40]
	Liver	Adult stem cells: ENR medium+FGF10+HGF+nicotinamide+gastrin+N-acetylcysteine Pluripotent stem cells: hindgut specification (activin-A+Wnt3a) → hepatic specification (FGF+BMP4) → Maturation (HGF+dexamethasone+oncostatin M) Embedded in Matrigel	Organ bud generation (closer to <i>in vivo</i>) Co-culture (cholangiocytes)	[41–44]
	Kidney	Wnt activation+FGF9 Suspension culture results in more nephrons than air-liquid interface culture	Vascularization Scalable, more mature (millifluidic system)	[45–51]

Wnt, wingless/integrated; ENS, enteric nervous system; EGF, epidermal growth factor; BMP, bone morphogenetic protein; FGF, fibroblast growth factor; HGF, hepatocyte growth factor.

Inhibition of transforming growth factor-beta and bone morphogenetic protein (BMP) signaling to prevent mesendodermal commitment results in definitive ectoderm formation and specification of the neuroectoderm. In 2011, Eiraku et al. [5] reported that prolonged embryoid body (EB) formation with the SFEbq method (serum-free culture of an EB-like aggregate with quick aggregation), followed by plating on coated dishes, resulted in complex structures resembling the developing cerebral cortex. Since neuroectodermal cells preferentially differentiate into the ventral hypothalamic progenitor, Lancaster et al. [6] reported a method to generate a “mini-brain” representing various brain regions without the use of growth factors. Embryonic stem cells (ESCs) were first aggregated into EBs and then embedded in Matrigel for the growth of neuroepithelial buds. The Matrigel droplets were transferred to a spinning bioreactor for enhanced nutrient absorption, which resulted in the rapid development of invaginated brain tissues composed of heterogeneous cell types surrounding a fluid-filled cavity resembling a ventricle.

The established cerebral organoids were later shown to recapitulate fetal neocortex development by Camp et al. [7]. Single-cell RNA sequencing showed that the changes in the single-cell transcriptome of the developing human cortex were similar to those observed in cerebral organoids. After developing cerebral organoids containing various brain regions, subsequent studies focused on generating region-specific spheroids [8,9]. Organoids or region-specific spheroids have been developed for other brain regions, such as the thalamus [10,11], choroid plexus [12], midbrain [13,14], cerebellum [15,16], and hippocampus [17–19]. Other advances have been made to generate brain organoid models more closely resembling the *in vivo* organ. Co-culture of different non-neural cell types with cerebral organoids, such as microglia [20–22], oligodendrocytes [21,23], and endothelial cells for vascularization, is the principal method used to improve brain organoids.

Another effort to improve the cerebral organoid model is the development of the assembloid. Brain assembloids were first developed to monitor the crosstalk between specific brain regions as they differentiate into region-specific cell types. Fused spheroids or organoids interacted with each other and extended functional axonal projections in a region-specific manner [9].

2) Retina

The retina originates from the neuroectoderm, protruding from the diencephalon to form the Rax-expressing optic cup. The multiple layers of the optic cup later develop into the pigmented outer layer and the inner neural layer, generating both rod and cone cells. The self-organizing optic cup organoid was the first

model entirely composed of neural tissue, where mouse ESCs and human ESCs (hESCs) were placed in minimal serum-free medium and Wnt inhibitors with dissolved Matrigel, which provided a scaffold to support the morphology of the optic cup [5]. The developing tissue showed characteristics of the optic vesicle, mainly the stratified levels of neuroepithelium with early retinal identity and the expression of retinal and epithelial markers. The model was improved further to create light-sensitive photoreceptors by extending the culture time and promoting maturation with all-trans-retinoic acid treatment [24].

The retinal organoid system itself is well-used to recapitulate organogenesis and retina-related diseases. However, the current retinal organoid system is limited, mainly because it loses crucial retinal ganglionic cells (RGCs) over time and lacks the retinal-brain connection. The assembly of retinal organoids with cerebral organoids ameliorated these shortcomings, where RGCs survived for over 150 days *in vitro* when fused with cortical organoids. The addition of thalamic organoids resulted in a shorter but larger number of RGC projections, showing that the triple organoid-fusion model better recapitulated the development of the retina *in vivo* [25].

Recently, Gabriel et al. [26] demonstrated that human brain organoids were able to develop bilateral optic vesicles when treated with retinoic acid for organoid expansion. Retinoic acid treatment resulted in the induction of a pigmented and invaginated area, which resembled optic vesicles in terms of marker gene expression. These optic vesicle brain organoids showed progressive maturation of the optic vesicles and developed early retinal cell populations, along with light sensitive-photoreceptors, which recovered after photobleaching and dark adaptation.

2. Endodermal organoids

1) Intestine

The intestinal epithelium has the highest turnover rate in adult tissues, heavily relying on actively cycling tissue-resident ASCs. The first organoid model was generated by Lgr5-expressing intestinal stem cells isolated from the small intestinal crypts, which exhibited an inverted form of the intestinal epithelium [2]. The small intestine originates from the definitive endoderm, to which hESCs can be differentiated with the treatment of activin A, and hindgut lineages can be derived by treatment with FGF4 and WNT3. The resulting organoid exhibited crypt-like features, intestinal stem cell markers, and CDX2-expressing intestinal mesenchyme [27]. Watson et al. [28] took another step to generate an *in vivo* model of the human small intestine

using hESCs via transplantation into immunodeficient mice, which resulted in significant maturation of intestinal epithelium. Even though mesenchymal cells are necessary for efficient differentiation and formation of a more mature organoid model, the surrounding mesenchyme must be removed by fluorescence-activated cell sorting (FACS) to study epithelium-specific factors, which complicates the experimental design for disease modeling. Therefore, subsequent studies sought to generate mesenchyme-free organoid models; for example, Mithal et al. [29] utilized induced pluripotent stem cells (iPSCs) grown in a serum-free medium with dual-suppressor of mothers against decapentaplegic inhibition, followed by cell sorting. The sorted population resulted in mesenchyme-free intestinal organoids, which exhibited mixed lineages, including the small intestine, colon, and liver. Optimization of the protocol succeeded in producing intestine-specific organoids, along with proof that intestinal organoids could propagate in the absence of a mesenchymal niche.

While ESC-derived intestinal organoids co-develop a mesenchymal layer, they lack ectodermal cell types required for full organ function, mainly the enteric nervous system (ENS). Recently, Workman et al. [30] mechanically aggregated human intestinal organoids (HIOs) and migrating neural crest cells (NCCs) by low-speed centrifugation and allowing them to grow in 3D growth conditions. This resulted in an HIO containing neurons and glial cells, which were rarely seen without NCCs *in vitro*. The established ENS showed periodical calcium efflux when stimulated, which led to smooth muscle contraction in HIOs, suggesting that the ENS was functional.

2) Colon (large intestine)

Isolation of human colonic stem cells was first documented by Jung et al. [31]. They isolated adult colon stem cells by FACS purification with epithelial markers and a known stem cell marker, EPHB2. The stem cells isolated with this method were able to propagate into 3D spheroids and, later, organoids with a single layer of colonic epithelium. Furthermore, the medium required for sustaining colonic organoids was very similar to that used for small intestinal organoids with a few critical changes, such as the addition of nicotinamide and prostaglandin E2, which led to a rapid loss of the upper crypt region and the formation of a closed spheroid with a central lumen. An alternative protocol was published by Sato et al. [32], who utilized their pre-existing protocol for small intestinal epithelium. A notable point of this protocol is that the addition of WNT3a inhibited proper differentiation into mature cell types but was still necessary for colonic organoid maintenance and stem cell self-re-

newal. James Wells' group first reported the differentiation of hPSCs into colonic organoids, where they were able to pattern hPSCs into SATB2-expressing colonic epithelium by transiently activating BMP signaling during early specification. In contrast, loss of BMP resulted in a significant loss of SATB2 expression. The BMP-patterned mid/hindgut spheroids maintained their distal colonic identity, as seen in the presence of a population of goblet cells only seen in the colon [33]. Crespo et al. [34] also developed colonic organoids from human iPSCs with a differentiation medium containing GSK3b and BMP inhibitors in the absence of serum, which produced pseudostratified epithelium with convoluted, crypt-like structures. These structures expressed the representative markers of colonic epithelium along with proliferating cells residing at the bottom of the crypt and minimal mesenchymal cells. The development of colonic organoids from PSCs has recently advanced. Park et al. [35] reported a method that generated colonic organoids containing enteric nerves or blood vessels by co-culturing nerve cells or endothelial cells with colonic organoids. The NCCs and endothelial cells used in that study were all differentiated from the same hPSC line as the colonic organoids, and integration into organoids was performed after the hindgut was defined from the definitive endoderm.

3) Gastric (stomach)

The stomach tissue develops from the definitive endoderm, like the intestine, but comes more specifically from the foregut instead of the midgut or hindgut. The generation of gastric organoids from hPSCs was made possible by McCracken et al. [36], who developed a protocol for the directed differentiation of hPSCs into gastric lineages, and later, gastric organoids. The derivation of pyloric tissue from mouse ESCs soon followed in 2015, when Noguchi et al. [37] established a protocol where embryoid bodies adhered to gelatin-coated dishes for development of the gut tube and transferred to Matrigel droplets with additional growth factors such as Dickkopf, SHH, Noggin, WNT3a, R-spondin, and epidermal growth factor. This led to endodermal tissues surrounded by mesenchyme, followed by the formation of a gut-like structure expressing more mature adult stomach markers. The generated tissue functionally produced pepsinogen-c and gastric acid when stimulated with histamine, and immunostaining revealed various cell types of the mature stomach, indicating the successful specification of the stomach by mimicking the *in vivo* differentiation of the gut tube. McCracken et al. [38] also engineered fundic organoids from hPSCs. They utilized a protocol for developing antrum organoids, but with the addition of Wnt activation via the GSK3b

inhibitor CHIR99021. This treatment was crucial for fundus development by repressing PDX1, a marker that separates the antrum from the fundus [39].

A recent attempt to establish an assembloid model of the intestine succeeded in generating a gastric assembloid, complete with surrounding mesenchyme, smooth muscle layers, and a functioning ENS. The mesenchyme layer promoted the growth of transplanted organoids, which do not usually survive when transplanted. The addition of NCCs induced glandular structures, smooth muscle, and myenteric plexuses upon transplantation into the kidney subcapsular space [40].

4) Liver

The liver is the largest internal organ in the body, and it plays an important role in maintaining homeostasis. It is also known for its ability to regenerate itself after injury and transplantation, so it is not surprising that the liver is one of the most extensively researched tissues for organoid development. The earliest studies conducted to generate liver-derived 3D structures were by Michalopoulos et al. [41], who isolated adult rat hepatocytes and other hepatic cells and placed them in roller cultures, which led to the formation of 3D structures with some properties of the liver. However, the architecture was very different from the current models of hepatic organoids, where the tissue covered the surface of the roller tube, and the cells could only survive in culture for a few weeks. Subsequent efforts to culture hepatic cells produced several protocols to differentiate hepatocytes from iPSCs [42,43], but it was not until 2013 that the current form of hepatic organoids was reported. Huch et al. [44] showed that Lgr5, a well-known stem cell marker in the gastrointestinal tract, was also expressed by hepatic stem cells that could be grown into 3D structures while maintaining their *in vivo* functionality and ability to differentiate into hepatocytes [45]. The same protocol could be used to generate self-renewing human liver organoids from liver biopsies, which exhibited genetic stability during long-term culture as adult ductal progenitor cells, while still being able to differentiate into functional hepatocyte-like cells *in vitro* and post-transplantation [46]. These protocols were adapted to allow long-term culture of mouse and human hepatocytes while maintaining their morphology and function. The critical factor was found to be tumor necrosis factor-alpha, an inflammatory cytokine, which promoted the expansion of primary hepatocytes in 3D culture [47,48].

Hepatic organoids were also developed from human iPSCs with patterning into hepatic endoderm and self-organization into 3D structures. The main difference between the organoid model and the “liver bud” model is the heterogeneity of the

liver bud, which better recapitulates the developing organ. The authors stated that organ-bud transplantation offers an alternative to the generation of a 3D, vascularized organ [49,50]. Alternatively, human hepatic organoid models containing both hepatocytes and cholangiocytes that could be used for disease modeling via CRISPR-Cas9 and hepatobiliary organoids that grew without exogenous cells or genetic manipulation and were capable of functional maturation when transplanted *in vivo* were developed and showed great promise in the field of regenerative medicine and transplantation therapy [51].

3. Mesodermal organoids

1) Kidney

The kidney develops from intricate interactions between the intermediate mesoderm, ureteric bud, and metanephric mesenchyme. Since the kidney is one of the most complex organs in terms of structure and lineage specification, and has a very limited capacity to repair itself, research on kidney organoids has focused on the differentiation of iPSCs as a means for transplantation therapy. Ureteric bud development was successfully recapitulated by Xia et al. [52], where hPSCs and iPSCs were patterned toward a renal lineage with BMP4, FGF2, activin-A, and retinoic acid. The differentiated cells successfully organized into 3D ureteric bud structures and spontaneously assembled into complex systems when co-cultured with mouse embryonic kidney cells. Taguchi et al. [53] developed a differentiation protocol to induce metanephric mesenchyme progenitors. Combining these findings, Takasato et al. [54] were able to direct hESCs into metanephric mesenchyme and ureteric bud lineages in an air-liquid interface culture, whose synchronous induction generated a self-organizing kidney. The same group further verified the established protocol. The kidney organoids contained characteristic kidney structures, such as individual nephrons, loops of Henle, and glomeruli [55]. The standard protocol for kidney organoids robustly produces kidney organoids. Nonetheless, the air-liquid interface culture system decreases epithelial cells and displays inefficient maturation after reaching a certain size. Therefore, the procedure was modified into a suspension culture system, where the organoids each contained up to 10 nephrons and showed a higher cell yield than the classic air-liquid interface protocol [56].

An important hurdle for kidney organoids is the lack of vascularization, which is necessary for the full function of the kidney and transplantation therapy. Van den Berg et al. [57] showed that *in vivo* transplantation of PSC-derived kidney organoids into the renal subcapsular space induces functional vasculogenesis.

esis, while endothelial cell loss is common *in vitro*. Extensive glomerular and tubular maturation could also be seen when transplanted *in vivo*, unlike *in vitro* culture, where prolonged culture does not affect organoid maturation. Due to the critical importance of vascularization for kidney organoid function, subsequent studies were conducted to develop a protocol that enables the scalability of organoid culture. One of these studies was done by Homan et al. [58], who developed a millifluidic system that allowed media to flow while the organoids were embedded in an extracellular matrix component. This current allowed the vascularization and maturation of kidney organoids *in vitro*, where it became possible to observe intra- and inter-organoid vascular network fusion under high flow. The media flow also enhanced cilia formation and polarization of the organoids, better recapitulating the *in vivo* kidney.

Disease modeling via cancer organoid generation

Unlike 2D culture, spheroids have a 3D structure, and enhanced interactions between cells. 3D cultured organoids exhibit more organ/tumor-specific features by introducing genetic mutations and TME. In recent research, more components are being in-

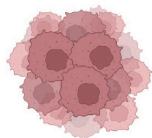
corporated to form multi-layered organoids called assembloids, which show the most similar characteristics to the corresponding *in vivo* structures, making them suitable for studying molecular pathways, epigenetic modifications, and drug screening. In the implementation of organ-specific characteristics, a tumor organoid is used to apply to disease modeling and precision medicine. Since the driver mutation causing the tumor and maintaining its phenotype is essential to reflect the characteristics of the organ, we will focus on the methods and characteristics of several tumor organoid model types. The major types of tissue-mimicking cell clusters are listed in Fig. 1.

1. Tumor spheroids

Spheroid models are scaffold-free models that are self-assembled from single-cell suspensions and used to model the behavior of various tumors, including breast [59], cervical [59], colon [60], lung [61], pancreas [62], and prostate [63] cancers. Spheroids can fill the gap between 2D and 3D culture techniques, mimic complex tumor scenarios such as the function and architecture of tissue, and increase the complexity of the model. The majority of techniques used today are low-cost and allow the reproducible production of a large number of spheroids [64].

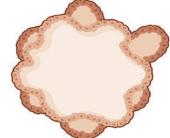
Homogeneous spheroid models are composed exclusively of

Spheroid



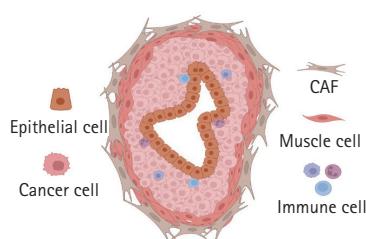
Scaffold-free model; cell aggregation
Filling the gap between 2D & 3D culture
Interactions with various cell types
Homogeneous/heterogeneous; multicellular spheroid

Organoid



Reflects characteristics of both tumor and normal tissue
Derived from tissue specimens/genetic modification
Diverse applications are available
(i.e. Studying tumor infiltration by co-culturing with normal and cancer organoids)

Assembloid



Multilayer assembloid; multilayer of cell components
Mimics tumor microenvironment
Assembloid generation by bio-printing technology
Advanced functional studies
(Molecular mechanisms, epigenetic changes, and drug screening)

Fig. 1. Characteristics of spheroids, organoids, and assembloids in tumor modeling. 2D, 2-dimensional; 3D, 3-dimensional; CAF, cancer-associated fibroblast.

isolated cancer cells, whereas multicellular spheroids contain both cancer cells and other cell types, including TME components such as fibroblasts, endothelial cells, immune cells, pericytes, and adipocytes [65]. The heterogeneity of these models facilitates tumor growth, progression, and metastasis [66]. Chen et al. [67] added cancer-associated fibroblasts (CAFs) to lung squamous carcinoma spheroids to observe cancer-specific pathological changes such as invasion and dysplasia. Ware et al. [68] incorporated pancreatic stellate cells into pancreatic ductal adenocarcinoma (PDAC) spheroids to form a dense and collagen-high pancreatic adenocarcinoma stroma spheroid, creating a model that mimics the actual tumor environment. Moreover, in heterogeneous spheroids, the stromal/cancer cell ratio can be optimized to mimic *in vivo* tissue even further [59].

2. Tumor organoids

Tumor organoids are powerful models used to study tumor initiation and growth, reflecting the characteristics of the tumor origin. They exhibit organ-specific cell types and functions, mimicking the architecture of the organ. They are established from patient tumor biopsy samples that can induce organ-specific phenotypic and genetic features, reflecting the distribution and differentiated cell types of the organ [69,70].

Long-term tumor organoid cultures have been established from a variety of tissues, including the colon [71], breast [72], liver [73], lung [74], stomach [75], prostate [76], ovary [77], bladder [78], brain [79], and bone [80]. Since cell and genetic manipulation is relatively easy in organoids, research on tumor suppressors or driver genes has been extensively conducted [81,82]. For example, introducing the *TP53* gene destroyed the tumor-suppressor locus, allowing spontaneous glioma development in a cerebral organoid model [83]. Malignant transformation can be induced by introducing individual mutations through genome editing, which can be utilized as a useful tool to study how specific genetic aberrations can affect the cancer phenotype. To model this interaction in a way that resembles *In vivo* conditions, establishing a TME component is necessary.

3. Cancer modeling using organotypic models

The TME plays a pivotal role in cancer, and interactions between cancer cells and TME components influence overall cancer progression and tumorigenesis, including migration, invasion, and angiogenesis. Furthermore, forming a heterogeneous microenvironment with specific patient stromal, immune, and structural components increases tumor heterogeneity. These studies make it possible to better understand the organ/patient-specific mutagenous process.

The TME can be represented through the co-culture of organoids and other cells. General organoids contain progenitor and epithelial cells, but no other stromal and immune cells exist. To overcome these limitations, various organoid-based models have been created by combining different cell types through co-culture systems.

Organoid models for studying cell-to-cell interactions through co-culture can be divided into 2 categories: co-culture with immune cells or CAFs.

In immune cell co-culture, tumoroids cultured from preserved tumors may be manufactured that include endogenous immune cells such as T, B, and natural killer cells, macrophages, and other non-epithelial cells [84–86]. As an alternative approach, research on the co-culture of extracted immune cell subsets with organoids is also being actively conducted [87,88]. Dijkstra et al. [84] confirmed the proliferation of tumor-reactive T cells in co-culture experiments with colorectal cancer and non-small-cell lung cancer organoids, and peripheral blood lymphocytes succeeded in specifically removing the tumor organoids, providing a means to evaluate the sensitivity of tumor cells to T cells. Neal et al. [89] also developed an air-liquid interface method to produce a patient-derived tumor organoid including stromal cells, native immune cells, and fibroblasts. Furthermore, tumor-infiltrating lymphocytes preserving the T cell receptor repertoire can be formed, and the immune checkpoint blockade between anti-programmed cell death ligand 1 (anti-PD-L1) and anti-PD-1 can be modeled.

Along with immune cells, CAF cells, as an essential component of the TME, are also extensively used in tumoroid co-culture systems to investigate the interactions between cell types [90]. Öhlund et al. [91] confirmed the differentiation of pancreatic stellate cells into CAFs by co-culturing them with PDAC, which affected PDAC progression and the therapeutic response, and demonstrated that the differentiated CAF cells were heterogeneous, with robust interactions between each CAF subpopulation. Seino et al. [92] confirmed that the critical Wnt niche of the TME was formed through interaction between PDAC organoids and CAF cells. Richards et al. [93] verified that co-culture of prostate stromal cells and cancer-derived organoids promoted the formation of branching, *in vivo*-like structures of epithelial organoids. The expression of alpha-methylacyl-CoA racemase was maintained and confirmed to have a positive effect on the viability of organoids.

Tumor progression can be studied through co-culture of normal organoids and cancer spheroids, as well as organoids and specific cell types, representative of which is infiltration research on glioblastoma (GBM) through the cerebral organ-

oid-GBM co-culture model. After establishing the first patient sample-derived GBM organoid, da Silva et al. [94] established a co-culture model of glioma stem cell (GSC) spheroids and immature brain organoids. This model made it possible to observe the infiltration process *in vivo*, study the molecular mechanisms of invasion, and screen for drugs that interfere with this process. The following year, Linkous et al. [95] successfully generated patient-specific GBM organoids derived from patient-derived GSCs and brain organoids using hESCs to study the invasion and proliferation of tumors residing in cerebral organoids. The co-culture of these 2 components made it possible to establish an infiltrative tumor model that mimicked the features of TME, as well as patient-specific characteristics in the cerebral organoid. The resulting tumor interacted with the TME network in the organoid, and it was confirmed that the accelerated invasion resembled the pathological features of surgical/autopsied specimens. This *ex vivo* model can be easily modified, both genetically and pharmacologically, and can be used as an effective tool to study the biology of GBM.

4. Tumor assembloids

Developing a tumor assembloid containing multiple tumor-related cells is closer to modeling the actual tumor. Kim et al. [96] developed a multilayer assembloid, including stromal components and muscle layers, to mimic the characteristics of the mature adult bladder, such as cell composition and generation. They also developed a tumor assembloid model with the pathological aspects of urothelial carcinoma, clearly reflecting the organizational characteristics of *in vivo* organs, such as the tissue stroma and microenvironment. The model showed features closer to those of the *in vivo* tumor, with an intricate vascular network and patient-specific genetic mutations.

The bladder cancer assembloid model also confirmed that the luminal to basal shift, which appears in long-term organoid maintenance, is inhibited by the appropriate stromal signals and re-demonstrated the importance of the stroma in maintaining the cancer subtype. Furthermore, it was revealed that enhancer reprogramming caused this subtype shifting by stroma-mediated epigenetic changes. Through this model, studying the molecular mechanisms and epigenetic modification of the tumor has become possible by comparing tumor assembloids, which more effectively mimic the *in vivo* tumors, with normal assembloids [96].

Present and future of organoid technology

In recent years, organoid technology has progressed exponen-

tially, as almost all types of cells have been used to generate organ/patient-specific organoids. A variety of organs in the human body have been successfully developed into organoids, including the brain, kidney, and the entire intestinal tract. The development of assembloids has increased our understanding of the interaction between several different tissues during development and took a step closer to establishing an “organ-in-a-dish.” The emergence of organoid biobanks enhanced the possibility of personalized medicine to be closer to reality [97]. Despite these advantages, the use of organoids to model *in vivo* organs is still limited, and there are several important considerations when utilizing organoids for research.

Excessively sacrificing laboratory animals to acquire cells/tissue is unnecessary for research using an organoid system. However, vascularization is essential for the culture of large organoids, and for modeling *in vivo* organs, transplantation into immunodeficient mice is inevitable. Even though organoids mimic many organ features, there are still significant differences that cannot be established *in vitro*. For example, the niche where stem cells reside in multiple cell types secretes specific factors necessary for its function and maintenance. Thus, it is evident that organoid models cannot fully replace animal testing.

Scalability is another issue for organoid culture systems. Many organoid models are based on Matrigel, an extracellular matrix extracted from mouse sarcoma cells that contains various scaffolding proteins and growth factors. Although Matrigel is very useful for maintaining the structure and function of many organoid models, it is difficult to handle and expensive, and its composition is not well defined, which results in high batch-to-batch variation. These features, along with the multiple growth factors and small molecules necessary for the sustenance of different organoids, limit the large-scale culture of organoids. In addition, patient-derived tumor organoids do not recapitulate the cancer genome and are generally not as scalable as neoplastic organoids; the scalability issue is also troublesome for cancer organoids [98].

As exemplified above, organoids are a versatile tool for recapitulating *in vivo* tissue, while simplifying tissue functions. Organoid technology can be used for basic organ biological research, disease modeling, transplantation therapy, drug discovery, toxicology, and other applications. Especially, the recent gene-editing techniques are widely used for disease modeling and correcting genetic defects. Various types of cancer organoids are being established and provide promising potential for cancer treatment, translational research, and drug development. For the further maturation of organoid technologies, many aspects such as vascularization, tissue engineering, interactions of

stem cells and their niche, and spatiotemporal control of signals should be considered. The extensive efforts currently underway should shed light on organoid technology development in the near future.

Notes

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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Data availability

Please contact the corresponding author for data availability.

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