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Recent Advances in Organoid Development and Applications in Disease Modeling

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Abstract

An improved understanding of stem cell niches, organogenesis, and disease models has paved the way for developing a three-dimensional (3D) organoid culture system. Organoid cultures can be derived from primary tissues (single cells or tissue subunits), induced pluripotent stem cells (iPSCs), or embryonic stem cells (ESCs). As a significant technological breakthrough, 3D organoid models offer a promising approach for understanding the complexities of human diseases ranging from the mechanistic investigation of disease pathogenesis to therapy. Here, we discuss the recent applications, advantages, and limitations of organoids as *in vitro* models for studying metabolomics, drug development, infectious diseases, and the gut microbiome. We further discuss the use of organoids in cancer modeling using high throughput sequencing approaches.

1. INTRODUCTION

A significant development in scientific methods over time has led to the generation of three-dimensional (3D) organoid culture and 3D printed scaffolds that can recapitulate human biology and diseases more precisely. In 1946, Smith and Cochrae first used the term ‘organoid,’ which means ‘resembling an organ,’ to describe a case of cystic teratoma [1]. However, now the term ‘organoid’ has a more restricted definition- i.e., organoids are self-assembled *in vitro* 3D structures, primarily generated from primary tissues or stem cells such as adult stem cells, induced pluripotent stem cells (iPSCs), and embryonic stem cells (ESCs). In all situations, the generation of organoids depends on the self-assembly and differentiation of cells and the signaling indications from the extracellular matrix (ECM) and the conditioned media [1]. Once the 3D structures assemble, they can mimic the complex

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Declaration of interests

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aspects of their organ counterparts, can be expanded long term, cryopreserved, and genetically modified.

Sato *et al.* developed the first 3D epithelial organoid in 2009 from a single leucine-rich repeat-containing G-protein coupled receptor (LGR5+) intestinal stem cell. The study showed that stem cells could be used to develop near-physiological, stable epithelial 3D structures when supplied with proper culture conditions (that mimic the *in vivo* stem cell niche) and embedded into a matrigel [2]. Later, organoids have been developed from various organs, such as the lung, liver, kidney, brain, pancreas, prostate, breast, taste buds, bladder, ovary, and stomach. Recently, *Mameishvili et. al.* has developed an organoid specifically from Aldh1b1 expressing mouse pancreatic cells and showed the Kras mutation, which is essential for pancreatic cancer progression [3]. They have also demonstrated the role of Aldh1b1 for tumor development in the mouse pancreatic cancer model [3]. Like organoids, spheroids are also multi-cellular 3D structures that are mostly used for studies related to cancer stem cells [4]. However, unlike the organoid culture system, spheroids are simple clusters of freely floating cell aggregates that are cultured under non-adherent, ultra-low attachment plates. Spheroids can be generated from cancer cell lines or tumor fragments, whereas organoids are specifically developed from tissue stem cells embedded within an ECM matrix. Another significant difference between the organoids and spheroids culture is the driving force's nature for their development. Organoids are formed mostly due to internal developmental processes, whereas spheroids are primarily developed via cell-to-cell adhesion [5]. Furthermore, spheroids contain a high proportion of poorly differentiated cells (a feature that indicates cancer stem cell characteristics), making them an excellent model system for cancer stem cell studies [5]. Since organoids represent a higher order of complexity and recapitulate their parent organ more nearly than spheroids, researchers are more inclined towards organoid technology for disease modeling and optimizing drug discovery and regenerative medicine [6]. Organoids have become a regular practice in the everyday lab, owing to their wide use in many biological research applications and the relatively fast pace of technological developments in this field. However, certain critical information is necessary before starting any organoid culture. Essential considerations are to choose the appropriate organ or cells (tissue chunks, iPSCs, or ESCs) and the suitable culture protocol, extracellular matrix, growth factors, and morphogens. Acquisition of in-depth knowledge pertaining to these considerations, together with an advancement in the protocols for culturing organoids, is bound to greater applicability of these 3D models in diverse fields. Here, we discuss the recent development in organoid culture techniques and the emerging applications of organoids in translational research. However, in this review, we did not particularly focus on a specific organoid; instead, we highlighted the currently known advancements in organoid culture techniques and evaluated its application in different biological fields.

2. ORGANOID CULTURE SYSTEM AND ITS INGREDIENTS

The major foundation for developing an organoid culture depends on the ability of the homogenous cells to undergo self-organization and reconstitute the structure and function of the tissue of origin. The process of self-organization can be divided into a series of self-patterning or cell differentiation processes that are highly regulated through various

morphogenic rearrangements. The traditional way of organoid development involves the digestion of tissue stem cells with collagenase, followed by culturing it in a 3D scaffold with growth factor conditioned media (Figure 1). Various morphogens, like Wnt-3a, R-spondin, epidermal growth factor (EGF), fibroblast growth factor (FGF), noggin, and gastrin are used for the culture of organoids (Table 1 and 2). However, a single specific media would not be helpful for culturing all different types of organoids from different tissue of origin. Therefore, several studies have used development biology research for deciding the media components according to the specific organ.

In addition to establishing appropriate growth-factor conditioned media, extracellular matrix (ECM) is another critical factor for organoid development. In the natural setting, the ECM is mainly composed of fiber-forming proteins, like collagen, elastin, laminin, fibronectin, and glycosaminoglycans, and release various growth factors, cytokines, and chemokines, thus regulates tissue development and homeostasis [7]. Therefore, while developing the 3D organoids model *in vitro*, it is necessary to mimic the ECM properties present in the tissue. Various matrices are available, from which the most commonly used matrix is 'Matrigel' (Figure 1C). The matrigel is a gelatinous protein mixture that resembles the ECM and is secreted by Engelbreth-Holm-Swarm mouse sarcoma cells [7]. It primarily contains a mixture of ECM components, including laminin, collagen type IV, entactin, heparin sulfate proteoglycans, and some growth factors such as TGFβ and FGF [7]. Another type of matrix that has been recently developed is 'Synthetic Hydrogels,' consisting of water-swollen networks made up of natural or synthetic polymers (Figure 1C) [8]. Compared to natural ECM gels, synthetic polymers are highly customizable and can be modified according to specific cultures by incorporating different proteins and other molecules. Recently, *Gjoresvki et al*. has also shown the potential of covalently cross-linked hydrophilic polymers (like PEG) hydrogels as an alternative to animal-derived 3D matrices, and they highlighted the effects of different ECM properties on organoid culture [9]. Despite this progress, synthetic hydrogels remain primitive compared to matrigel as they lack the critical property of cell-driven remodeling. In general, covalently linked hydrogels are inert and do not interact with cells unless they are not modified chemically. Integrin-binding peptides, such as Arg-Gly-Asp (RGD), has been shown to be grafted into the hydrogel networks to impart them with cell-adhesive property [10]. Since the synthetic hydrogels are constituted of the linear elastic network, they fail to recapitulate the physical characteristics of ECM, which lead to immature organoid growth and morphogenesis [10]. Furthermore, these covalently linked synthetic hydrogels' elastic network are often undergone irreversible breakdown in the covalent bonds, either through easter-based hydrolysis or MMP-based proteolytic cleavage, thus limit the long-term culture of organoids [10].

Finally, the characteristics of the organoid depend on the starting cell type. Studies have shown the establishment of organoids from ASC, iPSCs, or ESCs (Figure 1A). Different cell types follow different developmental pathways; therefore, choosing an appropriate starting cell-population is essential for successfully generating an organoid culture. Organoids generated from ASCs, or adult tissue fragments, have been shown to highly recapitulate the homeostasis and regenerative capacity of the tissue of origin [11]. However, organoids derived from iPSCs hardly recapitulate the adult tissue stage and resembles the fetal tissue stage [12, 13]. This characteristic of iPSCs-derived organoids makes them an excellent

system for studying developmental events and organogenesis. Interestingly, organoids of endoderm origin have been shown to be derived from both ASCs and iPSCs. On the other hand, organoids of ectoderm origin have mainly been generated from ASCs. Certain organoids such as cerebral organoids, neuroectodermal organoids, and mesodermal kidney organoids have only been shown to be derived from iPSCs [14]. When ASCs and iPSCs are highly used for the different organoids' generation, only a few studies have shown the use of fetal progenitors to derive the organoids [15, 16]. *Greggio et* al. has demonstrated the fetal progenitor-derived-pancreatic organoids from mouse fetal (embryonic age 10.5) dorsal pancreata [17]. Different germ layers in the embryo lead to the development of organoids with varying types of cells. This fascinating cell complexity can be used in vitro to understand the interaction among different cell types during the developmental process. In contrast, iPSCs-derived organoids resemble mostly the early post-implantation embryonic development, and the ASCs-derived organoids are mostly composed of a specific subset of cell types. Since the ASCs derived organoids are believed to mimic the adult tissue of origin, they are extensively used for disease modeling, like cancer and neurodegenerative diseases [18]. In comparison, iPSCs-derived organoids are mostly exploited to understand diseases related to developmental defects, such as microcephaly [18].

Though both ASCs and iPSCs derived organoids have their applicability and advantages, they both suffer from certain disadvantages. One of the critical challenges in iPSCs derived organoids is their maturation and scalability [19]. The organoids from iPSCs can only be grown to micrometer to millimeter scale due to limited culture conditions (such as oxygen and nutrient supply to the organoid core), thus limiting the life span and functionality of the organoids. Further, iPSCs-derived organoids are highly dependent on self-organization and require lengthy and precise protocols to generate appropriate progenitor cells, resulting in cellular heterogeneity and organoid-to-organoid batch variation [19, 20]. On the other side, organoids generated from ASCs were illustrated to be genetically stable when cultured for more extended periods [21, 22]. However, cancer-derived organoids have been shown to grow poorly compared with organoids generated from normal tissues or early stages of tumors [23]. Further, scientists have demonstrated the variability in optimal culture conditions between normal and tumor organoid cultures [23]. Another inherent problem with the technology is the requirement of matrigel for culturing the organoids, limiting the use of organoids in regenerative medicine [24]. In summary, the culture techniques for organoid generation from iPSCs or ASCs are still budding and are highly driven by empirical considerations.

3. BIOENGINEERING APPROACHES OF NEW GENERATION OF ORGANOIDS FOR DISEASE MODELING

The recent development in organoid culture techniques has made the organoid represent more molecular and physical similarities to their tissue origin. However, the organoid-based system constitutes some key challenges. One of the significant limitations is the restrained growth of the organoids and the development of the necrotic core. As mentioned above, iPSC-derived organoids reach only a specific maturation and resemble the fetal tissues. The phenomenon can be because of the lack of *in vivo* environment factors necessary for

organoid development. Different approaches have been initiated to recreate the tissue microenvironment, which can enhance tumor growth and development. In this section of the review, we are briefly discussing some of the bioengineering approaches for the better culture of the organoids (Figure 1B).

3.1 Bioreactors:

One of the major obstacles in organoid continuous growth is the proper diffusion of nutrients and oxygen to all the organoid cells. To overcome this, studies have exploited the bioreactor, whose constant spinning improves the aeration and nutrient uptake of the organoids in suspension culture [25–27] (Figure 1B (c)). Furthermore, the stirred-tank bioreactors have been shown to encourage more complex structures and increase the differentiation yield. A study using the brain organoid culture in the bioreactor system demonstrates that bioreactors can increase the organoid culture duration up to a year [25]. Other studies using the same system have yield iPSC-derived retinal organoids, kidney organoids, and brain region-specific organoids [26–28]).

3.2 Air-Liquid Interphase (ALI):

In an attempt to mimic the tumor microenvironment during the *in vitro* culture of organoids, scientists have developed the air-liquid interphase culture method (Figure 1B (b)). The technique involves the co-culturing of tumor epithelia with tumor fibroblast or with tumor-infiltrating lymphocytes [29]. In this method, the minced tumor tissue is resuspended in Type I collagen gel and layered on top of a pre-solidified collagen gel, *i.e.,* prepared on an inner transwell insert [29]. The culture media is present in the outer dish and diffuses to the collagen, i.e., present in the inner container via the permeable membrane. ALI culture system is mostly recognized and used for lung organoids [30].

Along with the respiratory organoid system, the method has been exploited for other organs, including the pancreas, renal cell carcinoma, melanoma, gastrointestinal tissue, and female reproductive tract [31, 32]. The significant advantage of the ALI method is its ability to incorporate both the epithelial and mesenchymal/stromal components, thus accurately recapitulating the stem cell niche in the organoid culture system [32]. Researchers further utilized this method to examine the *in vitro* interaction of tumor and immune cells in the tumor microenvironment [29]. However, it was demonstrated that immune and fibroblast stromal cells, when co-cultured with organoids through the ALI method, undergo apoptosis after a period of 1 or 2-month.

3.3 Vascularization:

Another major challenge in organoid culture is the lack of functional blood vessels, the absence of which leads to the development of immature organoids, the necrotic inner core, and premature differentiation. Different strategies involving sacrificial molding and laser ablation were explored to generate vascularized organoids [33, 34]. Both of these techniques allow the building of channels within the culture scaffold, which can host the endothelial cells to form a microvascular network. Another attempt to generate vascularized organoids involves the co-culturing of organoids together with vascular endothelial cells (Figure 1D (b)). This protocol has been successfully applied to generate vascularization in different

organ-specific organoids, such as the pancreas and liver [35]. Furthermore, the organ-specific vascular endothelial cells can be grown separately from the iPSC cells, and later the endothelial cells can be used to vascularize the organoid [35]. It was further shown that iPSC-derived brain organoids could be vascularized when implanted in the mouse brain [36]. When organoids were transplanted in different organs, such as the liver, intestine, and pancreas, they showed increased growth and maturation when exposed to *in vivo* factors [37–39] (Figure 1D (a)).

3.4 CRISPR/Cas9 genome-editing in Organoids:

Though in a very initial stage, scientists have started exploring the CRISPR/Cas9 technique to either knocking or knockout genes from organoids. Combining CRISPR/Cas9 with organoids can open new doors to understanding the role of specific genes in organ development and human diseases. CRISPR has been shown to be applied in mouse small intestinal organoids to cause frame-shift mutations in the two APC (known as a tumor suppressor and Wnt signaling negative regulator) alleles. The resulting organoids revealed growth in a Wnt-independent fashion. Further research has utilized CRISPR to introduce mutation in the KRAS, APC, p53, and SMAD4 genes in the colon cancer organoids [39, 40]. The technology has also been applied to iPSC-derived organoids. Freedman *et al.* had indicated that CRISPR could be used to model the disease phenotype. Their study generated a human iPSC-derived polycystic kidney disease (PKD) organoid and investigated the function by introducing truncating mutations in PKD1 or PKD2 genes [41]. Scientists have also started exploring genome-wide CRISPR screens in organoids. Ringel et al. have used the genome-scale CRISPR screening method in human intestinal organoids to investigate the TGF-β tumor suppressor pathway [42].

The CRISPR system can also be applied to introduce reporter sequences into the organoid genome. With the aid of CRISPR, fluorescent-labeled kidney organoids have been generated to uncover the role of nephron progenitor cells during nephrogenesis [43]. In the study, the researcher has developed the kidney organoids from the iPSCs cells that harbor Cre recombinase in the SIX2 locus and a loxP-flanked fluorescent cassette. Moreover, *Jung et al.* has used the same approach to visualize the different stages of intestinal differentiation in iPSC-derived intestinal organoids [44]. Though valuable, this technique has its limitation. Most importantly, the placing of the reporter gene, whose genomic location can hamper the gene expression. Although the technique has some limitations, it contains some major advantages. One of the significant advantages is to observe the differentiation of organoid cells in real-time. Further transplanting the reporter labeled organoid in the mouse can be tracked with fluorescence imaging [43, 44].

4. APPLICATION OF ORGANOIDS IN BIOLOGICAL SCIENCES

Following the establishment of organoids, the system has been exploited in different areas of biology and human diseases. For example, organoids developed from disease stem cells can be used to understand the genes involved and their molecular impact on disease progression study. Further, organoids can provide an excellent platform for investigating the viruses and

pathogens that infect the human body. Here we discuss various areas of biological sciences where organoids have found their extensive application.

4.1 ORGANOIDS IN METABOLOMIC STUDY

Recent pivotal studies support the notion that metabolic dysfunctions have a major effect on different diseases. Further, metabolic reprogramming at present is considered a hallmark of tumorigenesis. Advancements in 3D culture approaches have enabled organoids to study various genomic, transcriptomic, and proteomic alterations both at normal and disease conditions. We discuss the less explored application of organoids and their usefulness for studying cellular metabolism (Figure 2A).

Lu and colleagues demonstrated a culture protocol to establish 3D liver organoids for studying the hepatic glucose metabolism under normal and stress conditions [45]. Their work has shown that the hepatocyte organoids recapitulate the *in vivo* functions better than the hepatic monolayer or sandwich cultures [45]. The group has developed a hepatic bioreactor system, where the hepatic cells and collagen mixture was infused into hollow fibers to generate the liver organoids [45]. The developed liver organoids showed cuboidal-shaped hepatocytes and increased intracellular contact and bile-canaliculi-like structures. The same group also demonstrated that the entrapment of gel in the hollow fibers plays a critical role in the persistence of the cell functions [46]. The study suggests that the hollow fibers might mimic the blood vessels and provide a hepatocyte microenvironment for promoting hepatic cell assembly in collagen [46]. Another benchmark study by *Zietek et al.* has validated the use of intestinal organoids to assess nutrient transport, nutrient sensing, and hormone secretion [47]. Sodium-dependent glucose transporter SGLT1/SLC5A1, proton-coupled peptide transporter PEPT1/SLC15A1, and bile acid receptor TGR5 in the apical brush border side and facilitated glucose transporter GLUT2 in the basolateral side of the intestinal organoid. Altogether, *Zietek et al.* highlighted the robust applicability and usefulness of organoids in the field of metabolism.

Organoids have also been implicated in studying stem cell niche metabolism in the small intestine. For instance, researchers have developed mini-guts organoids with defined crypt domains and Wnt-conditioned organoids (resembling embryonic small intestinal organoids) to study mitochondrial oxidative role phosphorylation during differentiation and crypt formation [48]. *Rodriguez-Colman et al.* have used the standard media (containing epidermal growth factor, noggin, and R-spondin1; ENR) and Wnt3a-conditioned media (WENR) to generate the mini-gut organoids and Wnt-conditioned organoids, respectively [48]. Additionally, using the same organoid model, the study demonstrated that the glycolytic phenotype in Paneth cells and mitochondrial oxidative phosphorylation in Lgr5+ crypt base columnar cells is necessary for supporting both niche and stem cell function [48]. In another study, the use of human pluripotent stem cell-derived cardiac organoids (hCOs) was reported to explore the maturation of the mammalian hearts during postnatal life [49]. The hCOs model revealed that the metabolic switch from carbohydrate to fatty acids acts as a critical driver for cardiac maturation [49]. Organoids have also been exploited to examine the role of amino acids on stem cell functions and growth. *Sean et al.* investigated the critical function of L-alanyl-L-glutamine (a stable L-glutamine (Gln) dipeptide) in maintaining

intestinal epithelial homeostasis [50]. Sean and co-authors have shown that Gln promotes intestinal stem cells' expansion and mTOR signaling in jejunum-derived mouse organoids. Furthermore, *Saito et al.* illustrated the effect of deficiency of essential amino acids on enteroid stem cell differentiation to intestinal enteroendocrine cells, enterochromaffin cells, goblet cells, and Paneth cells [51]. Human liver organoids are composed of multiple hepatic cell types: epithelial hepatocyte cells, hepatic stellate cells, and kupffer cells have been established from human pluripotent stem cells. Using this model system, it was illustrated that free fatty acid exposure to the liver organoids leads to increased inflammation and triggers a physiological condition known as stenosis [52].

With the recent emphasis on learning the metabolomics of cancer stem cells using 3D organoid models, various matrigel-based extracellular flux analysis bioassays were developed [53] [54]. These assays help to determine the crypt metabolomic profile by measuring the oxygen consumption and extracellular acidification rates of the organoids and thus provide an excellent opportunity to define the role of metabolism during tumorigenesis. Furthermore, imaging techniques such as optical metabolic imaging (OMI) have been developed to provide an attractive non-destructive platform to quantify cellular metabolism in organoids. OMI utilizes the NAD (P)H and FAD autofluorescence intensity and a lifetime to detect the metabolic states of the cells. Using this method in breast, pancreatic, and head and neck tumor organoids, researchers have shown the metabolomic changes of organoids in response to cancer drugs [55–57]. To further explore the distribution of drugs and their metabolites, matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) system was used in patient-derived colon tumor organoids [58]. The use of such mass spectrometry-based approaches to study metabolic profiles in 3D has led to the emergence of a new era of organoid research that encompasses its application to high throughput technologies, as discussed in the next sections.

4.2 ORGANOIDS FOR GUT MICROBIOME STUDY

Studies have shown the potential of organoids for understanding the dynamic interaction between diet, microbiota, and the host intestinal epithelium [59, 60] (Figure 2B). The gut microbiota, which consists of trillions of microorganisms in the gastrointestinal tract, plays significant functions in determining human health. These microorganisms have been shown to regulate the lifespan, bioavailability, chemical structure, and biological activities of the ingested compounds via diet. Besides, microbial metabolites have been shown to facilitate multiple intestinal signaling pathways, thus making this metabolic-microbiota and host interaction a potential target for developing therapeutic drugs [61]. To date, the effect of microbiota on host metabolism has been studied mostly in human 2D cell lines. Since 2D monolayer cultures minimally recapitulate the cellular complexity and organization, 3D organoid models were used to enumerate the role of microbial metabolism on host disease pathogenesis. Lukovac and colleagues have examined the influence of microbially produced short-chain fatty acids on intestinal function by using mouse gut organoids [62]. The bacterial-conditioned media for culturing the organoids were used to prove the concept. The different gut-microbiota members stimulate different responses in the organoids by modulating the genes involved in various metabolic pathways [62]. The microbially generated short-chain fatty acids play a crucial function in maintaining gut health [63].

Interestingly, intestinal organoids have shown their potential to study the beneficial effect of gut microbiota-derived short-chain fatty acids on mitigating graft-versus-host-diseases [64]. Additionally, *Schilderink et al.* determined the impact of short-chain fatty acid, butyrate, inhuman, and mouse intestinal organoids [65]. The activation of ALDH1A1 and ALDH1A3 expression through butyric acid plays a vital role in converting epithelial retinol to retinoic acid, thus maintaining gut homeostasis [65]. Furthermore, the intestinal organoid system has addressed the role of enterochromaffin (EC) cells as chemosensors that detect stimuli from ingested chemicals, gut microorganisms, and endogenous regulatory pathways [66]. Similarly, Sarah and co-authors have investigated the function of microbially-derived acetate, propionate, and butyrate on intestinal stem cells using duodenum organoids [67].

Organoids have also been implicated in studying the influence of diet on tumorigenesis. A study of flavon-3-ols (extracted from grape) on human and mouse-derived colon tumor organoids showed the inhibitory effect of flavon-3-ols on colorectal cancer [68]. Flavon-3-ols available to the gut-microbiota and are metabolized to low-molecular-weight phenolic products [69–71]. Another very recent study has highlighted the gut microbiome's role in determining a cancer mutation [60]. The study found contrasting effects (both tumor-initiating and tumor-suppressive) of mutant p53 in different segments of the gut and mouse-derived tumor organoids. However, he showed that supplementing the organoids with gut microbiota-gallic acid can suppress the tumor-suppressive effect of p53 and lead to the tumor phenotype in organoids.

Even though 3D organoid models provide an excellent platform to ascertain the role of gut-microbiome and host interaction, this technology has several challenges. First, the healthy and disease-derived organoids' responses can be different and should be taken into account. Further, the microbes interact with the apical epithelial surfaces, i.e., the lumen which faces the interior of the enteroids. Thus, for evaluating the gut microbially-derived metabolites in intestinal organoids, the polarity of the cells should be considered. To achieve this, organoids are disrupted into single or clumps of cells followed by embedding into the extracellular matrix and directly culturing with the media containing the desired microorganism. Since dissociating the organoids expose the apical side of the cells; therefore, it provides a suitable interaction between the microorganism and the intestinal organoid cells [72]. Another technique is microinjection, which employed the introduction of microbes into the organoid lumen (Figure 2B (i)). However, the collection of cell debris and mucus within the enclosed organoid lumen can make this technique confounding and labor-intensive. A new technique has been introduced to overcome this problem, which reverses the apical polarity of the organoids by manipulating the extracellular matrix composition. In this current method, the organoids were first developed in matrigel equivalent basement membrane extract [2] and then transferred to suspension culture growth media (in low attachment plate) without dissociating the organoid into single cells [73]. Using this technique, the authors showed that the organoids in the suspension culture lack a lumen, and thus the boundaries of epithelial cells become indiscernible.

Studies have suggested that most of the microbial communities in the gut environment are comprised of anaerobic microbes, which are highly sensitive to oxygen. Hence, the high throughput microinjection technique was used to study the interaction of anaerobic microbes

with the intestinal organoids. In a recent study, the authors have developed a semi-automated high-throughput microinjection device that transports the microbes or cargo directly into the lumen of the organoids [74]. Since the organoid lumen is satisfactorily hypoxic, the authors showed the survival of an oxygen-sensitive anaerobe, *B adolescents,* in the organoid lumen over four days.

Altogether, these studies corroborate the concept that intestinal organoids recapitulate the gut microenvironment and, therefore, can serve as a suitable model to examine the microbe-gut-host interaction.

4.3 ORGANOIDS FOR INFECTIOUS DISEASES

For many decades, single-cell culture and animal models have provided the stage for understanding the host-pathogen interaction under disease conditions. These models also advance the overall development of drugs and vaccines for treating infectious diseases. However, the absence of complex cellular context and the inherent differences of the immune system between humans and animals limit the use of single-cell culture and animals. Advancements in 3D organoid cultures have boosted drug development and helped understand infectious diseases and host-cell responses (Figure 2C). One of the uses of organoids in infectious diseases involved the study of Zika virus infection (ZIKV) using the brain organoid. In 2016, the ZIKV was infected into the human pluripotent stem cell-derived cerebral organoids to comprehend its association with neurological complications in newborns. Using brain organoid, ZIKV was found to induce neural cell death in the early stages of brain development [75]. Further, *Gabriel et al.* showed that ZIKV induces premature differentiation of infected neural progenitor cells and generates mitotic defects [76]. *Dang et al.* had used human embryonic stem cell-derived organoid to recapitulate the early stage of fetal brain development and demonstrated a mechanistic connection between disrupted neurogenesis and ZIKV mediated TLR3 activation [77].

Organoids, such as intestinal organoids, have shown their robustness for the assessment of noroviruses and rotaviruses infections [78, 79]. A study using intestinal organoids infected with noroviruses showed bile as a critical factor for strain-dependent noroviruses replication [78]. Human-derived small intestine and lung organoids were exploited to define the *Cryptosporidium* infection mechanism. *Cryptosporidium* is a protozoan parasite and is the leading cause of diarrhea and child mortality worldwide. To understand the pathophysiology of *Cryptosporidium*, *Heo et al.* have analyzed the *Cryptosporidium* transcriptome during its infection and demonstrated that *Cryptosporidium* could replicate in the organoids and completes its entire life cycle [80].

Organoids are also highly used for understanding the infections related to the respiratory tract. Generally, the first contact of pathogenic microbes and the respiratory tract is through epithelial cells, and epithelial cells can be easily grown as organoids. During the COVID-19 pandemic, lung and gut organoids are highly used for understanding the SARS-CoV-2 infection and biology. Studying SARS-CoV-2 with intestinal organoids showed that the angiotensin-converting enzyme 2 (ACE2) receptor (through which the virus enters the cell) is highly expressed in the brush border of intestinal organoids. Furthermore, it was found that the SARS-CoV-2 virus can infect both high and low-expressing ACE2 cells. One of the

early studies by *Lamers et al.* has demonstrated that airway and gut organoids can be used as a model to study the SARS-CoV2 infection in the lung and gut [81]. The study revealed that SARS-CoV2 mainly targets and infects the ciliated cells but not the goblet cells. Additionally, a recent study has utilized the human iPSC derived brain organoids to understand the direct involvement of SARS-CoV2 in the central nervous system [82]. The authors detected increased viral antigen (SARS-CoV2 nucleocapsid) protein and viral particles in the cryosectioned brain organoids. The localization of SARS-CoV2 to TUJ1- and NESTIN-positive cells of the brain organoids were also shown, thus suggesting the direct infection of cortical neurons and NPCs SARS-CoV2. Furthermore, another group of researchers has developed region-specific brain organoids to investigate and understand the ability of SARS-CoV2 to infect and impair the function of brain cells [83]. Though the adverse outcome of COVID-19 is respiratory failure, often, many of the patients develop additional complications involving gastrointestinal, neurological, and metabolic systems. A retrospective study on 7,337 COVID-19 patients has shown a strong correlation between the poor outcomes in COVID-19 patients and type 2 diabetes [84]. Liuliu and the group have generated liver and pancreas organoids to investigate the viral tropism of SARS-CoV2 [85]. This organoid-based study provided evidence that pancreatic alpha and beta cells and liver organoids are permissive to SARS-CoV2 infection. *Zhao et al.* has shown the infection of liver ductal organoids by SARS-CoV2 virus and demonstrated increased cholangiocyte damage upon SARS-CoV2 infection [86]. Organoids were also used robustly to study the efficacy of drugs that are developed against SARS-CoV2. A recent study has highlighted the effect of clinical-grade soluble human-recombinant ACE2 in blocking the growth of SARS-CoV2 infection in human blood vessels organoids and kidney organoids [87].

Besides this, an ACE2 (+) expression was studied in multiple organoids using single-cell RNA sequencing analysis. The analysis revealed that ACE2 is highly expressed in intestinal, lung, and retinal organoids, and limited-expression was found in brain and prostate organoids [88]. Further, SARS-CoV2 causes multiple organ damage, with acute kidney injury (AKI) as the second leading organ damage in COVID-19 patients. Xia and the group have established long-term cultures of normal human kidney proximal tubule epithelial cells (KPTECs) by co-culturing the KPTECs organoid with irradiated J2 fibroblast feeder cells to investigate the infection of SARS-CoV2 in kidney cells [89]. Another study in human long organoids has tested the therapeutic potential of 25-hydrocholesterol (25HC) against SARS-CoV2 infection [90]. In COVID-19 patients, SARS-CoV2 infection stimulates the cholesterol 25-hydroxlase gene, which then mediates cholesterol conversion to 25HC. 25HC was found to inhibit the viral membrane fusion and thus block its entry into human lung organoids.

Organoids have also been used to understand the human reproductive tract infections, such as herpes simplex virus type 2 (HSV-2), chlamydia, gonorrhea, syphilis, and human immunodeficiency virus (HIV) infection [91]. HSV-2 infection in females predominantly occurs in the lower female reproductive tract (FRT). The mucosal lining of the vagina, which is made of stratified squamous epithelial cells, interacts with the FRT pathogenic organisms. A significant challenge to study infectious diseases is the recreation of a microenvironment of native tissue. The air-liquid interface (ALI) organoid cultures of vaginal epithelial cells were developed to overcome this problem and determine the mechanism of HSV-2 infection

[92]. The study focused on examining the effect of progesterone on the cells of the vaginal epithelial organoid when they were exposed to HSV-2 infection. When compared to the traditional liquid-liquid interphase culture of vaginal epithelial cells, ALI closely mimics the in vivo condition of the FRT compared to liquid-liquid interphase, therefore provide a better model to study the host-pathogen interaction in FRT. Recently, Kessler and the group have utilized the human fallopian tube organoids to analyze the genital *Chlamydia trachomatis (Ctr)* infection and its effect on epithelial homeostasis [93]. Furthermore, the author elucidated an increase in LIF signaling and increased stemness in the organoids when infected with *Ctr*. Moreover, DNA methylation pattern changes of the genomic DNA from the infected organoids showed that *Ctr* enhances the DNA hypermethylation. These studies highlight progress in the organoid culture establishments for investigating the essential aspects of host-pathogen interaction during infection. Moreover, the list of pathogens that can be grown and organoids to study their infection mechanism is still growing.

4.4 ORGANOIDS FOR TUMOR MICROENVIRONMENT

The *in vitro* cultures of neoplastic cells as an organoid typically consist of epithelial cells and matrigel that mimic the tumor microenvironment. However, a significant part of the solid tumor comprises the tumor microenvironment, involving tumor-infiltrating immune cells, vasculature, and tumor-stroma. Several studies have demonstrated the mechanism for tumor microenvironment involvement in tumor growth. However, there is a dearth of *in vitro* models that reiterate tumor cells and tumor microenvironment interaction. Advent in organoid culture opens new approaches to understanding tumor microenvironment in cancer (Figure 2D). Using ALI methodology, it has been demonstrated that culturing patient-derived organoids with tumor-infiltrating lymphocytes can successfully recapitulate the PD1/PD-L1-dependent immune checkpoint. Co-culturing cancer-associated fibroblast (CAFs) with tumor cells as a single stroma-attached organoid is another method to understand the involvement of tumor microenvironment in cancer. The method involves the aggregation of dissociated tumor cells and CAF and the matrigel and put as a single dome in a cell-culture well plate [94, 95]. *Seino et al.* used the single stroma-attached organoid to reveal physical attachment or the juxtracrine interaction of CAFs with pancreatic ductal adenocarcinoma cells (PDAC) is necessary for CAFs to support the growth of PDAC organoids under Wnt3A deprived condition. In addition, co-culturing of PDAC organoids with pancreatic stellate cells discloses the presence of two distinct CAF subtypes, characterized by either IL-6 expressing inflammatory CAFs and a-smooth muscle actin (a-SMA) expressing myofibroblastic CAF [95]. IL-1 and transforming growth factor (TGF)β are identified as the tumor-secreted ligands, which promote the distinct inflammatory and myofibroblastic subtype [95]. In addition, DeNardo and the group utilized mammary submerged organoid cultures to investigate the involvement of the adaptive immune system ($CD4^+$ T cells) in macrophage-induced mammary invasive behavior [96]. Treatment of non-invasive mammary organoids with tumor-associated macrophages (TAMs) (isolated from mammary carcinomas) and T_H2-type cytokines (IL-4 or IL-13) resulted in organoid disruption and formation of invasive structures. Further, to demonstrate the direct involvement of $CD4^+$ T cells in regulating TAMs induced mammary tumor cell invasion, the aforementioned culture was tri-cultured with $CD4^+$ T cells. Another study of PDAC organoids and CAFs and peripheral blood lymphocytes co-culturing has shown the

lymphocyte infiltration and movement of the myofibroblast-like CAFs, towards the tumor cells [97].

Tumor-induced fibroblast support tumor growth in breast cancer; however, much less is known about the role of normal fibroblast in tumor progression. Organoid co-cultures were initiated with estrogen receptor-positive (ER+) breast cancer cells with normal breast fibroblasts (NAF) and tumor-associated fibroblast (TAF) [98]. Co-culturing the breast tumor organoids with NAFs revealed the tumor-induced secretion of pro-proliferative factor IL1β from NAFs. Furthermore, the co-culturing of organoids with TAF led to the secretion of CCL7 from TAFs, thus enhancing the release of PDGF-BB from the tumor cells, which then induced the secretion of IL1β from NAFs [98]. Overall, it described the paracrine signaling involvement between ER+ breast cancer cells, NAFs, and TAFs that boosted the ER+ breast tumor cell proliferation. Co-culturing of human endothelial cells with mouse breast organoids has shown the development of a functional capillary vessel network that could unite to mice circulatory system [99].

Furthermore, current organoids protocols are depending on animal-derived matrigel or collagens for the matrix. However, both collagen and matrigel are composed of complex components, such as collagen IV, entactin, laminin, and various growth factors. The presence of these undefined growth factors can influence the growth and cellular activity of the organoids. Therefore, researchers have identified and established new generation matrices with known components to increase the potential application of organoids.

4.5 ORGANOIDS FOR THERAPEUTICS AND DRUG DEVELOPMENT STUDIES

According to precision medicine, every patient should be treated differentially based on their genetic and transcriptomic landscape. However, failure to understand individual patients' landscapes leads to treatment failures or resistance towards the drug. The majority of the disease modeling and drug screening studies involved using the 2D monolayer cultures as their model system; however, 2D cell lines exhibit multiple culture-induced mutations or contaminations with other cell lines. This has fueled organoid's increased application in early drug discovery programs and toxicity screens [49]. Furthermore, patient-derived organoids can be generated rapidly even with a limited amount of harvested tissue, and it highly maintains the heterogeneity and genetic features of their original tissues.

Organoids developed from the patient's tissue samples can also be used for personalized treatment regimens (Figure 2E). Growing the matched healthy and diseased organoids from patient tissue samples allows the clinical screening of various drug combinations that will efficiently and specifically target the diseased tissue [51]. In cystic fibrosis, the drug effectiveness depends on the mutational type of the CFTR gene. However, characterizing the mutation and the specific drug treatment for the cystic fibrosis patient is time-consuming and costly. *Dekkers et al.* demonstrated that rectal organoids could be useful in predicting cystic fibrotic patients who can potentially respond to the drug [100]. Furthermore, the Bear group has utilized CFTR patient-derived respiratory organoids for examining a fluorescent-based assay, which measures the patient-specific drug responses based on the CFTR gene mutations [101].

Organoids biobank has been established for various types of tumors: colon, pancreas, prostate, and liver cancer. Using the two biobanks derived from colon cancer was exploited to screen 83 clinically used drugs [102]. *Vlachogiannis* and the group have developed live organoid biobanks from patients with metastatic gastrointestinal cancer and compared the drug responses of the organoids with the patient response in the clinical trials [103]. Further research on patient-derived pancreatic cancer organoids has investigated the sensitivity of chemotherapeutic drugs based on gene expression signatures [104]. Moreover, the same study has reported new driver mutations in the KRAS wild-type pancreatic tumors that involve ERBB2^{S310F}, MAP2K1$^{Q58\text{-}E62del}$, and PIK3CA^{E110del}. The authors suggested alternative targeted treatment regimes, such as afatinib (for ERBBS310F mutations) and everolimus (for PIK3CAE110del mutations) for chemo-refractory pancreatic organoids. Screening of chemotherapeutic agents; temozolomide (TMZ) and bis-choloethylnitrosourea (BCNU), and ionizing radiation on glioma stem cells (GSC) and cerebral organoid glioma (COG) showed high resistance activity of GSC towards drug and radiation-induced damage [105]. However, when GSC was co-cultured with 2D cerebral cells exhibited more genotoxic stress on drug and radiation treatment [105]. Overall, the study revealed that COG recapitulates the *in vivo* disease condition and treatment responses more closely than 2D cultures. *Phan* and the group have established a mini-rings geometry assay system to perform high-throughput drug screening of 3D tumor organoids [105]. The method involves the establishment of organoids in a ring shape around the rim of a 96-well plate. The assay provides the advantage of changing the media or addition of drugs to be easily performed by pipetting directly in the center of the well, thus preventing the disruption of the gel. Using this technology, *Phan et al.* has tested 240 kinase inhibitors (FDA approved or in clinical development) in patient-derived organoids and suggested specific drugs to individual patients. A study using colon organoids has shown the effectiveness of afatinib and other EGFR inhibitors in advanced-stage colon cancer with APC mutation [106]. Foundation Hubrecht Organoid Technology (HUB; Utrecht, The Netherlands) had conducted a multi-centered cohort study on organoids derived from the biopsy of the metastatic colon, breast, and non-small cell lung cancers. The study demonstrated a positive correlation between the drug responses on the tumor organoids with clinical responses of patients [107]. EGFR inhibitors were also explored in pancreatic cancer by Kaushik et al. [108]. In the study, authors have utilized pancreatic tumor organoids to demonstrate the selective inhibition of pancreatic tumor stem cells by afatinib (a pan-EGFR inhibitor) and gemcitabine combination treatment. Further, it was shown that afatinib and gemcitabine synergistically decrease metastasis in pancreatic cancer.

Another benchmark study in drug discovery using the organoid system was presented by *Greka et al.* research. Greka's group has identified misfolded mutant mucin 1 (MUC1-fs) protein trapped in the tubules of Mucin 1 kidney disease patient's organoids [109]. With this knowledge, the same group has identified a potential drug, BRD4780 (a small molecule), to treat Mucin 1 kidney disease. As a mechanism, it was shown that MUC1-fs is trapped in TMED9 cargo receptor-containing vesicles, and treatment with BRD4780 leads to the release of MUC1-fs by binding to TMED9.

The major limitation in drug screening is the differences in experimental protocol and the approaches for data analysis. The development of a more standardized screening and data

analysis method can increase the robustness of drug-screening data. To achieve this, *Driehuis et al.* have provided an in-depth protocol for establishing head and neck squamous cell carcinoma (HNSCC) organoids and a subsequent semi-automated therapy screen method [110]. By improving the traditional organoids protocol, Arlotta's group has generated long-growing organoids that resemble the rich diversity of cell types of the human cerebral cortex [111]. With this improved protocol, the Arlotta group suggests that brain development's dynamic process can be modeled in organoids and can provide a valuable model to investigate developmental abnormalities associated with human neurological diseases. This type of work put the foundation to understand the genetic alterations related to neurological diseases and the development of drugs to target these mutations.

Organoids are just not limited to cancer, other notable diseases such as polycystic kidney disease, microcephaly, psychiatric disorders, and aforementioned infectious diseases can also be studied through this model system.

4.6 ORGANOIDS IN HIGH THROUGHPUT SEQUENCING APPLICATIONS

Organoids serve as a 3D platform to model difficult to study processes temporally and spatially. Examples include the onset of neuropsychiatric disorders in the human fetal brain, early epigenomic changes leading to cancer initiation, among other developmental pathologies. The organoid model transcriptome and epigenome study help identify functional elements that may facilitate disease onset [112]. Furthermore, organoids function as a translational bridge between *in vitro* and *in vivo* models, enabling rapid transition of *in vitro* discoveries into the clinics. This is particularly relevant for drug screening in case of aggressive malignancies wherein administering a drug suited to the patient's genetic profile is critical for better prognosis and disease management. Organoids, therefore, can play a central role in personalized medicine. Given organoids can essentially function as miniature organs in a dish, evaluating the transcriptome and epigenome of these *in vitro* models through high-throughput platforms such as analyses at the single-cell level would enable us to dissect development and pathophysiology comprehensively. Recent advances in the methodology to isolate and culture tissues as three-dimensional (3D) organoids have led to profound improvements in the success rate of deriving organoids from normal and diseased human tissues. However, it remains unclear if organoids can precisely recapitulate the cell state-specific transcriptomic and epigenomic landscapes of the tissues intend to model.

With recent advances in high throughput and omics profiling technologies, it is now possible to characterize organoids at the transcriptomic, proteomic, and epigenomic levels (Figure 3). Such high throughput applications, both bulk and single-cell sequencing technologies. While bulk sequencing applications have provided many insights through comparison of normal and diseased states, single-cell applications hold tremendous potential to be of use in areas that require understanding cellular heterogeneity. In addition, intercepting the proteomic milieu of physiological and disease states using mass spectrometry is gaining momentum in recent years [113]. In the section below, we discuss some of the recent applications of single-cell RNA-seq and other epigenomic technologies in organoids. We discuss the challenges and future applications of the intersection of the 3D culture and high-throughput single-cell technologies in various tissues.

Gut: Most applications of the single-cell RNA-seq have been restricted to mouse small intestine organoids to date, and these studies have provided the most insight into the cellular composition of adult stem cell-derived organoids [114–117]. Grun et al. sequenced the transcriptome of hundreds of randomly selected cells from mouse intestinal organoids to identify rare cell types from the intestinal epithelium [116]. An algorithm called RaceID for rare cell type identification in complex populations of single cells. Using this algorithm, Reg4 was identified as a unique marker of a rare population of hormone-producing enteroendocrine cells. Not only did RaceID confirmed the existence of known enteroendocrine lineages, but it also discovered novel subtypes, which were subsequently validated in vivo. A rare population of Lgr5-positive secretory cells within the ex vivo-isolated Lgr5-positive stem cells and their direct progeny were identified. In another study, the mouse intestine atlas was generated by profiling 53,193 individual epithelial cells from the mouse small intestine and organoids [117]. Through single-cell RNA-seq, the authors could classify as cell type or state as enterocyte, goblet, Paneth, enteroendocrine, or tuft cells. A new enteroendocrine cell taxonomy was generated by comparing genes in each cell cluster with the canonical classification markers. Interestingly, heterogeneity in the tuft cell population was split into two distinct subtypes Tuft-1 and Tuft-2. Tuft1 and Tuft2 cell populations were enriched in unique signatures related to neuronal development and immune-related genes. Furthermore, the authors compared cell composition and gene expression landscapes in organoids after exposure to different microbe populations, providing insights into how cell types and gene expression profiles in the intestine evolve with microbial infections. In another seminal study, Biton et al. applied single-cell sequencing analysis and organoid culture to understand the contribution of adaptive immunity in intestinal stem cell maintenance [118]. A nonconventional antigen-presenting role of $Lgr5^+$ intestinal stem cells and the influence of Th cells on epithelial cell renewal was deciphered. They found that $Lgr5^+$ intestinal stem cells interacted with activating naive Th cells via major histocompatibility complex class II (MHCII) antigen presentation. Treg and interleukin-10 (IL-10) were found to promote the renewal of the intestinal stem cell compartment, while Th1, Th2, and Th17 cells and derived cytokines (i.e., IL-13 or IL-17) depleted intestinal stem cells and induced organoid differentiation. In addition, luminal signals (bacteria or parasite) influenced the intestinal stem cell-Th axis and shaped epithelial cell composition. This study is a classic example of how epithelial and immune cell crosstalk can act as a regulated response to maintain intestinal stem cell self-renewal or promote differentiation. The aforementioned studies were conducted using murine organoids. Extended to humans, single-cell RNA-seq will allow controlled experimentation of human intestinal cell types to diverse dietary, microbial, or pharmaceutical manipulations. However, improved organoid culture methods are needed to enable the long-term culture of progenitors and differentiated cells isolated from the human gut within a 3D structure.

Liver: Generating liver in vitro from pluripotency via recapitulating the growth pathways has been challenging due to the dynamic developmental, structural, and cellular heterogeneity of the organ. iPSCs in 2D monocultures can be differentiated to hepatic endoderm and then toward ‘hepatocyte-like’ cells [119, 120]. However, these ‘hepatocyte-like’ cells do not function as mature, metabolically competent hepatocytes. Cells with only modest similarity to human hepatocytes have been generated using certain widely used

protocols, which may even be off-target cells with similarity to the intestinal epithelium [121]. Incorporating additional signals such as the mesoderm-derived paracrine signals that promote hepatocyte maturation in liver organoids and a 3D microenvironment that mediates cell-to-cell surface contact has shown great promise in generating hepatic organoids [37, 122]. It has been shown that 2D monocultures of primary adult human hepatocytes can only be maintained short-term due to dedifferentiation and cell death [123]. However, with the advancement in protocols for isolation of hepatic stem cells (HSCs) from adult tissues and culture HSCs in 3D matrix environments, the differentiated hepatic cells can be maintained as genetically stable organoids [22]. Furthermore, primary liver tumor organoids have successfully been established using tumor resections from liver cancer patients [124].

Broutier et al. showed that the primary patient-derived liver cancer organoids maintain the expression profile of the corresponding tissue-of-origin and tumor subtype using genome-wide transcriptomic (RNA-seq) analysis [124]. Each tumor organoid was subjected to whole-exome sequencing (WES) analysis following short (<2 months, early passage) or extended (>4 months, late passage) periods in culture to ascertain whether the different organoids retain the parent tumor's mutational landscape. The organoids retained >90% of the patient's tissue variants for early cultures and >80% after long-term culture to compare the global variant profile. Further, the single nucleotide variants (SNVs), indels, and the distribution of base substitutions in the original tissue were retained in culture. Additionally, novel genes with prognostic value in liver cancer cohorts were identified through comparative gene expression profiling of tumor organoids versus healthy liver organoids. These findings demonstrate the potential of high-throughput analyses conducted using organoids to identify novel genes of importance in human liver cancer. Single-cell RNA-seq analyses on the adult liver could, in principle, map the transcriptome states of the hepatic progenitors, hepatic stem cells, and mature hepatocytes in vivo, which can be compared to the transcriptome of the adult stem-cell-derived organoids maintained *in vitro*. Such comparisons would enable assessment of the accuracy and precision of organoid culture *ex vivo* and would reveal the dynamic changes in the transcriptome of these organoids with time. In any case, current protocols lack cellular diversity (e.g., kupffer cells, stellate cells, bile ducts, portal endothelium, etc.) and the structural organization of the human liver. In the future, modifications to the existing protocols to support the *in vitro* growth of diverse hepatic cell populations would lead to liver organoid technology benchmarking.

Pancreas: Applications of the organoid culture to the pancreas include the study of *in vitro* differentiation of pancreatic progenitors to insulin-producing islets and the pathogenesis of pancreatic cancer [15]. Several studies indicate that epigenomic reprogramming is an emerging mechanism of pancreatic ductal adenocarcinoma (PDAC) progression. Inactivating mutations of chromatin modifiers are frequent genetic events in pancreatic tumors [125]. Further, an association between the disruptions of large heterochromatin domains with the metastatic transition in pancreatic cancer has been demonstrated [126]. Given the importance of evaluating the epigenomic landscape changes during pancreatic cancer progression, organoids may serve as a robust and representative miniature *in vitro* models of studying pancreatic cancer metastasis. Roe et al. used organoids generated from primary pancreatic tumors (T) and matched metastatic (M) tissues derived from the autochthonous

PDAC mouse model (Kras$^{+/}$LSL-G12D; Trp53$^{+/}$LSL-R172H; Pdx1-Cre) [127]. The authors found regions with consistent GAIN and LOSS of H3K27 acetylation in all M organoids compared to matched T organoids, irrespective of the anatomic site of the metastatic lesion. These GAIN regions with enhanced H3K27 acetylation, indicative of enhancer elements, were enriched at the corresponding locations in human PDAC cell lines and patient-derived PDAC organoids. Ontology analysis of genes located near GAIN regions revealed a significant enrichment of developmental functions and implicated FOXA1 in GAIN enhancer activation. Through high throughput approaches such as H3K27ac ChIP-seq and ATAC-Seq, the authors showed that FOXA1 mediated enhancer reprogramming drives PDAC aggressiveness, and manipulation of FOXA1 alters enhancer activity in PDAC cells and turn affects metastasis. This study underscores that applying high throughput approaches in organoids can lead to significant strides in uncovering disease pathogenesis mechanisms. In another study, patient-derived PDAC organoids were injected into the pancreatic duct of immunocompromised mice using a modified retrograde pancreatic intraductal injection method [128]. The authors referred to this *in vivo* model as the intraductal grafted organoid (IGO) model. Tuveson and colleagues had previously generated another *in vivo* model of PDAC that comprised injecting the patient-derived PDAC organoids directly into the interstitium of immunocompromised mice (referred to as an orthotopically grafted organoid model or OGO) [129]. Since PDAC progression is believed to occur in the pancreatic ductal system, the IGO model is better at recapitulating the progression from preinvasive cells within the duct system to extra ductal frank invasive tumors surrounding desmoplasia. To determine the influence of the different microenvironments on the transcriptome of the tumors, the authors performed RNA sequencing (RNA-seq) using pairs of tumors derived from the OGO and IGO organoids. The OGO xenografts were shown to exhibit the previously described “squamous PDAC” [130] and “basal-like” [131] gene signatures. In contrast, the IGO xenografts were enriched in “progenitor PDAC” [130] and “classical” [131] signatures using high throughput transcriptional and molecular analyses. Moreover, xenografts derived from the organoid models were classified as Fast- and Slow-progressor phenotypes correlated with the molecular analyses and subtype classifications of human PDAC. This study is a classic example of how organoids can be used as personalized models of PDAC and can provide insights into the heterogeneity of the disease.

Brain: *In vitro* neural differentiation from induced pluripotent stem cells (iPSCs) and human embryonic stem cells (hESCs) recapitulates early brain development stages starting with the initial commitment to neuroepithelium followed by the sequential creation of neural progenitor cells and subsequent birth of early and late neurons. Complex structures in the developing brain, such as the progenitor zones along the apical-basal axis and a diverse variety of functional neurons, can be generated in organoids [132, 133]. Because human brain development is a highly dynamic process driven by a spatially and temporally controlled transcriptional program that functions synchrony with an epigenomic program, cerebral organoids serve as an ideal model for examining *in vitro* neural differentiation. Luo et al. used whole-genome methylome profiling and transcriptome sequencing to examine the epigenomic and transcriptomic changes during *in vitro* differentiation using cerebral organoids and the human fetal brain [134]. Transcriptomic signatures in organoids faithfully modeled gene expression profiles in the early-to-mid human fetal brain. The authors also

found that regions with Non-CG Methylation overlapped with super-enhancers and were actively expressed during early fetal stages but became suppressed after the transition to postnatal development. Further, the cerebral organoids showed enrichment for common transcription factor binding motifs and promoter DNA methylation signatures as that of the human fetal brain. Overall, the remodeling of the methylome observed during cerebral organoid differentiation in this study underscored that the epigenomic state achieved in the cerebral organoids closely resembled the human fetal brain [134].

5. CONCLUSION

The accessibility and the property to imitate *in vivo* tissue biology have made the organoid a great platform to study different fields of disease modeling. This model has shown its potential for research, including therapeutic responses and drug discovery. Although there is immense development in the technology for 3D organoid culture systems, the researchers are still exploring and standardizing techniques for their robust use in translational research. A major limitation in the field involves that the researcher has no control over the function and behavior of the cells once they assemble into organoids. This drawback limits the use of organoids to full potential. However, collaborating with advanced bioengineering techniques allow the culture of organoids in a more defined environment. Bioengineered biomaterials and microtechnology can mimic the embryo environment by providing controlled geometric and/or mechanical inputs to the organoids. Moreover, utilizing the engineering technology makes it possible to deliver morphogens/growth factors in a controlled manner. These approaches will impart more systemic information in the field and lead to the defined growth of organoids. Finally, scientists are also exploring the bioreactor, ALI culture method, and vasculogenic technologies to address the microenvironment and overcome the nutrient availability problem. Given the rapid advance in organoid technology, we trust that organoids will provide better opportunities to study and be used in human health.

6. FUTURE PROSPECTS

Despite the extensive application of organoids in the various biology fields, there are still several diseases where the organoid culture technology has not been explored yet or is still in infancy. Most of the neurodevelopmental or neuropsychiatric conditions, such as schizophrenia, Parkinson or autism, are mostly studied using animal model systems [135]. The disorders like autism spectrum disorders or Parkinson’s constitute clinical heterogeneity (including epilepsy, sleep disruptions, and motor disturbances), making it difficult to investigate using an organoid culture system [136–138]. However, researchers have started developing protocols to generate more mature and complex brain organoids that can be actively used for neuropsychiatric studies [139]. Furthermore, organoids can be used to understand diseases, such as developmental brain injury and disorders (DBD) related to the early developmental stages of the brain. A lot of the diseases have been shown to be associated with stem cells. However, scientists are still confounded by how the anomalies are happening within the stem cells or how they know which type of specialized cell it has to make. Therefore, the organoids system can be used to answer the involvement of stem cells in a disease like emphysema, a type of lung disease, where lung stem cells fail to repair the damage [140]. Further, scientists have suggested the idea of using organoids to screen drugs

that can enable the formation of specialized cell types for genetic diseases, like cystic fibrosis, where ciliated cells that eradicate mucus from the lung are not working appropriately [141]. Organoids from cystic fibrosis patient tissues can be generated, followed by developing a drug that might make the ciliated cells work better in the organoid culture system. Since scientists have developed the techniques to co-culture the organoids with different immune cells, organoid culture technology can also be explored to understand the mechanisms and screen drugs for autoimmune diseases. Moreover, the application of organoid technology can be exploited for immunotherapy studies on tumor organoids, where T-lymphocyte from a healthy donor can be activated and expanded with patient-derived organoids. Organoids generated from pluripotent stem cells mimic the early embryonic organs, therefore, contain both epithelium and mesenchymal cells [20]. Recent studies on organoids have been used to address the fibrosis mechanism in the lung and intestine [142]. Using the same protocol, organoids can also be used to study fibrosis in other organs, such as the liver and kidney. Though in a very initial stage, scientists have already started thinking of applying organoid technology in the above-mentioned diseases.

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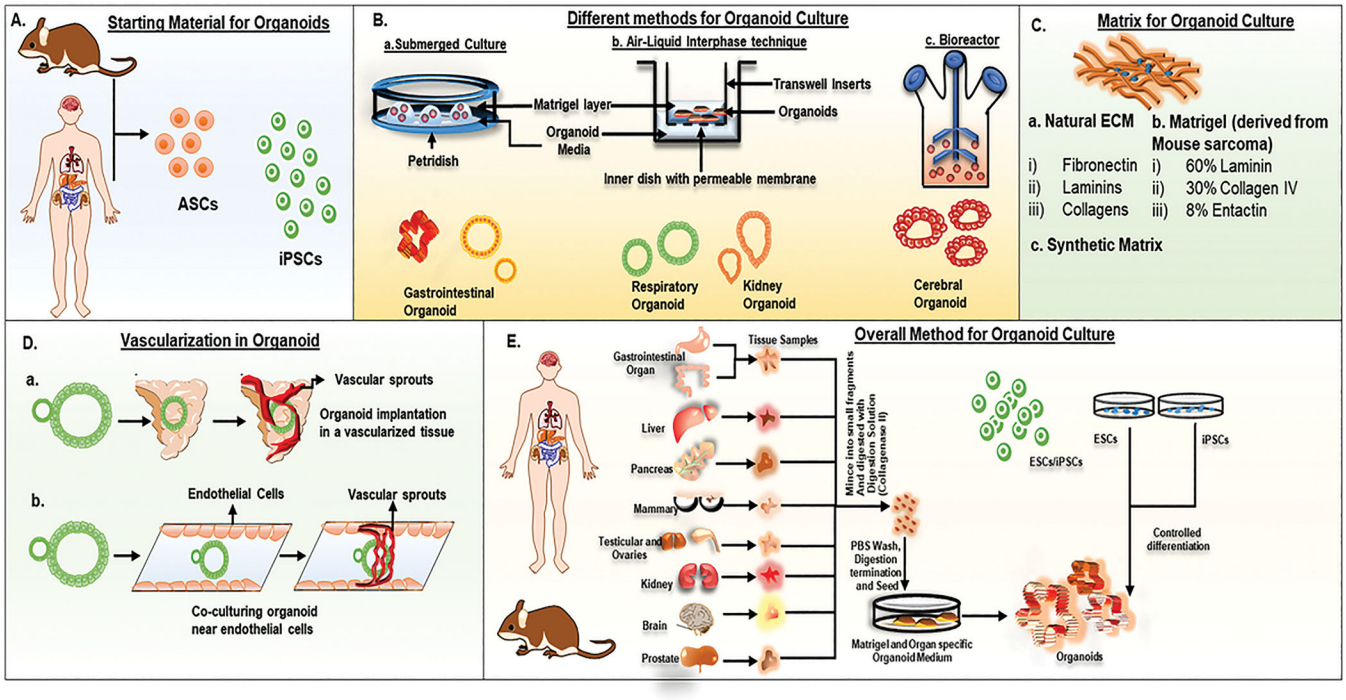

Figure 1: Overview of culture techniques and bioengineering advancements for an organoid generation:

A. Organoids can be generated from adult stem cells (ASCs) isolated from human/animal tissue samples or induced pluripotent stem cells (iPSCs). **B.** The culture method is important for the proper development of organoid. The essential methods are (a) submerged culture, (b) air-liquid interface, and (c) bioreactor technique. The submerged culture method is widely used for the generation of organoids of gastrointestinal origin. In the ALI method, organoids are grown on an inner dish, and the media is placed under the inner dish. This technique is widely used for the development of respiratory and kidney organoids. Further, this technique is also explored for the interaction study of organoids with the tumor-microenvironment. The disadvantage of submerged culture and the ALI method involves the limitation of nutrient availability once the organoid reaches a specific size. This limitation is overcome by bioreactors (3D suspension culture) that enhance the organoid's availability of nutrients. This technique is mostly explored for cerebral organoids. C. Matrix is another important material that propelled the self-organization of cells towards a specific structure and led to organoid growth. Promising matrices that are well explored for organoid generation are: (a) natural extracellular matrix (ECM), (b) Matrigel; derived from mouse sarcoma, and (c) synthetic matrices. Bioengineering advancements have designed various synthetic matrices, which provide spatiotemporally controllable 3D matrices that can be exploited to give the desired architecture to the organoids. **D.** Vascularization of organoids can provide a near-physiological way to provide nutrients to the organoid cells. To common techniques explored for the vascular network in organoids are (a) implantation of organoids in a vascularized tissue and (b) co-culturing the organoids in between two layers of endothelial cells, which further lead to the generation of vascular sprouts in the organoids. E. Explains the overall approach for the generation of organoids from tissue sections or iPSCs/ESCs. In iPSCs/ESCs, the cells are first to undergo controlled differentiation to specific

organ cell types and then followed by mixing with matrigel to form organ-specific organoids.

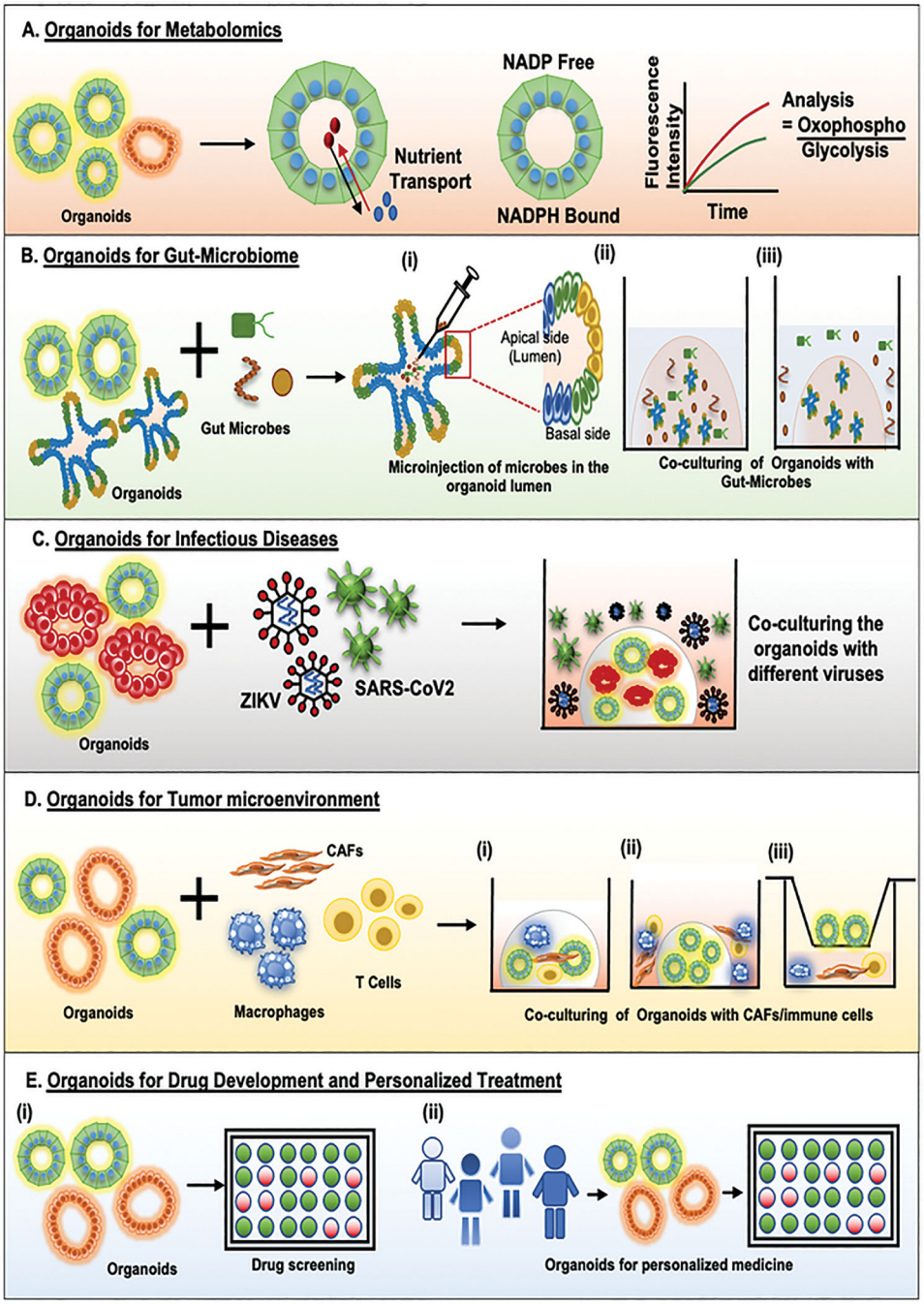

Figure 2. Application of organoids in different biology or biomedical sciences:

A. Organoids can be used to understand the metabolomics regulation or nutrient transport mechanism of cells under disease or normal conditions. **B.** Intestinal/gut organoids could be used to study the effect of gut-microbiome on cell physiology and growth. Different culture techniques are explored to study the gut-microbiome in organoids: **(i)** Microinjection involves injecting the gut-microbes or gut-microbiome-derived metabolites in the apical surface of the organoids. **(ii)** Mixing the organoids and gut-microbes with matrigel and culturing them together in the ECM matrix. **(iii)** Co-culturing the organoids with gut-

microbes by putting the microbes in the organoid medium. **C.** Organoids represent useful tools for the study of infectious diseases through co-culturing the diseases related viruses with the organoids. **D.** Organoids provide a good platform to understand the role of cell niche or microenvironment in cell growth and differentiation. Techniques such as **(i)** embedding the organoids together with tumor microenvironment cells in the matrigel or **(ii)** adding the microenvironmental factors in the culture media or through **(iii)** Air-liquid interphase were explored to study the cell-niche along with organoid. E. **(i)** In drug screening and **(ii)** personalized medicine, patient-derived organoids can help to identify the specific/best drug for a specific disease or each patient.

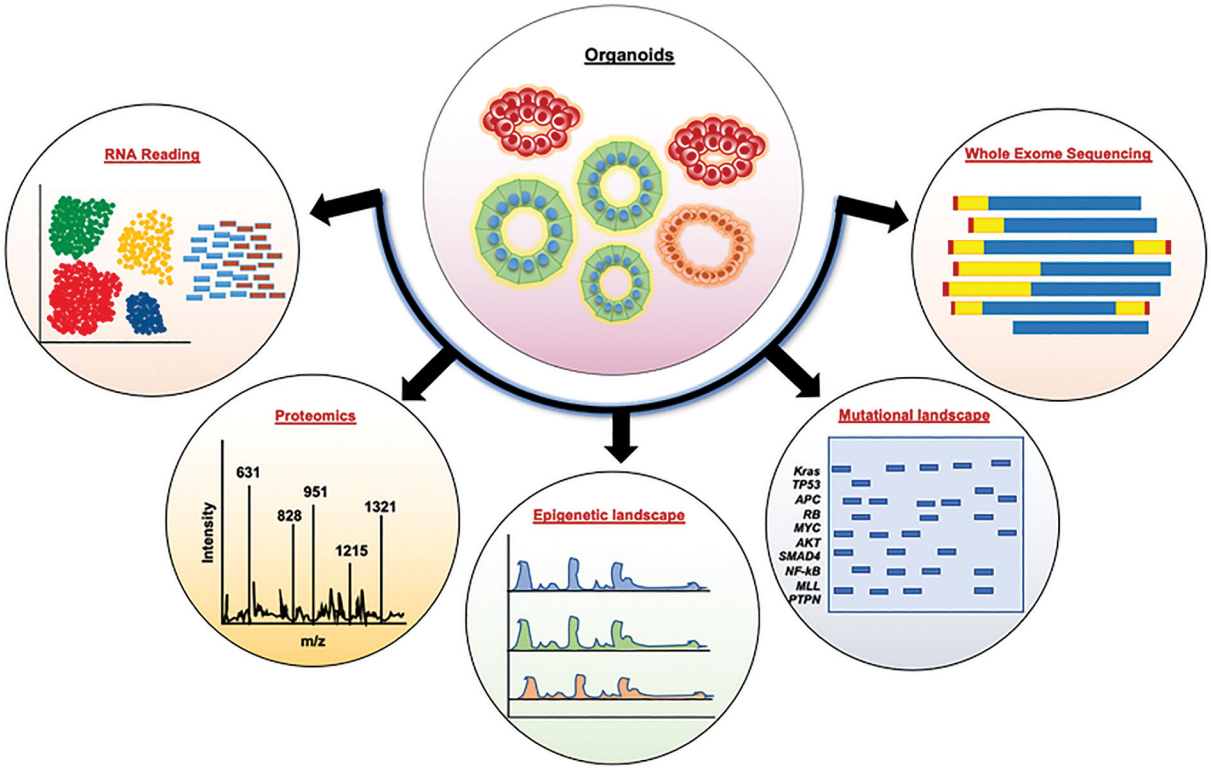

Figure 3. High-throughput sequencing approaches in organoids:

High-throughput sequencing approaches have been extensively explored in organoids to study different molecular mechanisms during cancer or other diseases. **(i)** RNA-sequencing and single-cell RNA sequencing in tumor organoids are used to profile the total transcriptomic status of the cells during tumor development or metastasis. **(ii)** Proteomics analysis on organoid through mass-spec illustrate the changes in various proteins during disease vs. normal. **(iii)** ChIP-Seq or ATAC seq analysis in the organoids demonstrate different epigenetic profile. **(iv)** Patient-derived organoids are extensively used to study the mutational landscape or load during tumor development. Various mutations such as Kras, TP53, APC, etc., and their oncogenic functions have been profiled in tumor organoids. **(v)** Whole-exome sequencing in organoids is used to understand the changes or mutations in the protein-coding regions of the genes.

Table 1:

Summary of specific media compositions for different organoids

Cerebral Organoids	Intestinal Organoids	Liver Organoids	Pancreas Organoids	Prostate Organoids	Lung Organoids	Mammary Organoids
Neurobasal-Medium NEAA Ratinoic Acid R Spondin Noggin	DMEM/F12 FBS EGF R Spondin Noggin Wnt	DMEM/F12 FBS EGF R Spondin FGF HGF Nicotinamide Insulin	DMEM/F12 FGF EGF B27 R-Spondin FGF Nicotinamide Noggin Wnt A-8301 Gastrin	DMEM/F12 FBS EGF R Spondin Noggin Dihydrotestosterone	DMEM/F12 FBS B27 N2 GlutaMAX FGF Noggin SB431542 CHIR99021	DMEM/F12 FBS ITS-Seliniem X EGF Hydrocortisone T3 β Estrdiol Isoproterenol Hydrocholoride Ethanolamine O-phosphorylethanolamine

Ovary & Fallopian tube Organoids	Kidney Organoids	Bladder Organoids	Esophageal Organoids
DMEM/F12 FBS R-Spondin FGF EGF Nicotinamide Noggin Wnt	DMEM High Glucose FBS NEAA GlutaMAX Hesparin APEL media FGF9 SB431542 CHIR99021	Hepatocyte Media EGF FBS GlutaMAX Primocin	DMEM/F12 EGF Noggin R-Spondin Wnt SB202190 A83–01 Nicotinamide Gastrin

Table 2:

Summary of functions of growth factors and small molecule inhibitors applied in organoid cultures.

GROWTH FACTORS	FUNCTIONS
EGF	• EGF stimulates the proliferation by binding to EGF receptors. • EGF induces hyperplastic changes and stimulate proliferation of cancer cells.
FGFs	• FGFs are a group of signaling protein play crucial role in normal development. • FGFs are mainly mitogens and also alternately known as pluripotent and promiscuous growth factors due to their multiple functions on multiple cell types.
Wnt	• Wnt plays a major role in embryonic development by its main functions on axis patterning, cell fate specification, cell proliferation and cell migration.
Noggin	• Noggin play a role in germ layer-specific derivation of specialized cells and acts as a regulator of bone morphogenetic proteins (BMPs) during development.
R-Spondin	• R-spondin proteins are secreted agonist of Wnt/β-catenin signaling pathway.
Gastrin	• Gastrin act as a growth factor in organoid culture and stimulates the proliferation of cells.
A83–01	• Functions as a potent inhibitor of TGF-β I receptor superfamily and inhibits the differentiation.
Nicotinamide	• Nicotinamide is a type of vitamin B3 and act as part of the coenzyme nicotinamide adenine dinucleotide.
SB202190	• Function as a p38 inhibitor
CHIR99021	• A small molecule inhibitor of GSK3 signaling molecule, and thus promotes proliferation and growth by increasing the β-catenin and c-Myc protein stabilization.
Y27632	• A Rho kinase inhibitor that inhibits the anoikis of dissociated stem cells.