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3D cell culture: from evolution to revolution

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Abstract

Recent advances in the isolation of tissue-resident adult stem cells and the identification of inductive factors that efficiently direct differentiation of human pluripotent stem cells (hPSCs) along specific lineages have facilitated the development of high-fidelity modelling of several tissues in vitro. Many of the novel approaches used have employed self-organising three-dimensional (3D) culturing of organoids, which offer several advantages over conventional two-dimentional platforms. Organoid technologies hold great promises for modelling diseases and predicting the outcome of drug responses *in vitro*. Here, we outline the historical background and some of the recent advances in the field of 3D organoids. We also highlight some of the current limitations of these systems and discuss potential avenues to further benefit biological research using 3D modelling technologies.

Key words: Pluripotent stem cells, organoids, 3D culture

Introduction

The isolation and maintenance of mammalian cells have significantly advanced scientific research into cellular processes and mechanisms of disease that include stem cell development and differentiation, the production of monoclonal antibodies, and therapeutic proteins and for modelling cancer *in vitro* (1). Although culturing tissues dates back to the late nineteenth century, present cell culture systems draw from studies on the action of serum on fibroblast cells (2) and the development of novel synthetic cell culture media (3, 4). A classic example of this was the isolation and expansion of HeLa cells from a cervical tumour on a two-dimensional (2D) monolayer culture (5).

Since then, culturing cells in 2D has remained the predominant methodology of in vitro cell growth and expansion. However, the 2D platforms do not effectively recapitulate the spatial requirements that are essential for the organisation and cellular interactions, which occur *in vivo*. In addition, it is suspected that limited cell-cell contact and altered *in vitro* cell signalling networks can result in major discrepancies between the data acquired from 2D in vitro versus *in vivo* research.

Historical background

To overcome 2D platform limitations, efforts have led to development of novel approaches to recreate a more physiologically relevant environment in the form of 3D cell culture (1). To successfully construct and maintain a 3D structure, much research has been devoted to the development of synthetic or natural polymeric 3D scaffolds to facilitate cell growth. These efforts have resulted in the fabrication and characterisation of several non-degradable or biodegradable synthetic polymers such as poly-lactic acid, poly-glycolic acid, poly-lactic-co-glycolic acid and poly caprolactone (6). Initially, a “top-down” approach was adopted where cells were seeded on a prefabricated scaffold, however, difficulties in recreating the intricate microstructural characteristics of tissues have remained the major limitation of this approach (7). Later, “bottom-up” assembly of small cellularised blocks and layer-by-layer assembly (also known as 3D printing) have been developed (8). In this review, we focus on scaffold-free methods to culture cells in 3D and the generation of organoids by embedding cells in semi-solidified extracellular

matrices (ECM) in contrast to the use of polymeric scaffolds and 3D printing, which have been reviewed extensively elsewhere (6, 9, 10).

Techniques to generate scaffold-free 3D cellular aggregates

In general, scaffold-free approaches rely on cell-cell interaction and can be categorised into passive or active methodologies. The passive methodologies solely rely on cell adhesion properties, in which cells require time to form solid aggregates (11). Various passive methods have been developed to generate scaffold-free 3D aggregates robustly and consistently (**Fig. 1**).

Hanging-drop is the first technique to generate 3D structures by culturing suspended droplets of desired cell line(s) to force aggregation (**Fig. 1a**). In fact, Robert Koch et al. invented the hanging-drop methodology in the 1880s to grow anthrax bacilli in a suspended drop of fluid taken from oxen eyes in a special concave microscope slide (12). Later, this method was adopted by Harrison and co-workers to monitor nerve outgrowth (13). Harrison's pioneering work led to the development of various techniques for short-term culture of dissected tissues during the early 20th century. Although 3D spheroids can be generated efficiently using this technique, the lack of scalability promoted the development of high-throughput culture methods that use 384-hanging drop arrays, which are amenable to automation (14, 15).

Later, the liquid overlay method was developed to generate 3D microtissues on non-adherent surfaces. Using this method, random interactions of cells resulted in the formation of large numbers of spheroids, which were usually heterogeneous in size (16, 17). As the nutrients and oxygen exchange is based on passive diffusion in static culture, formation of necrotic centres in large spheroids is a major drawback of this methodology (18). To improve consistency and control, the size of formed microtissues, micromoulds (**Fig. 1b**) and patterned microplates have more recently been used (19).

More advanced methodologies have also been developed for large scale production of 3D microtissues, which include, spinner flasks, rotating wall vessel bioreactors, and microfluidic systems (**Fig. 1c**).

a. Hanging drop

b. Microplate mould

c. Stirred flask culture

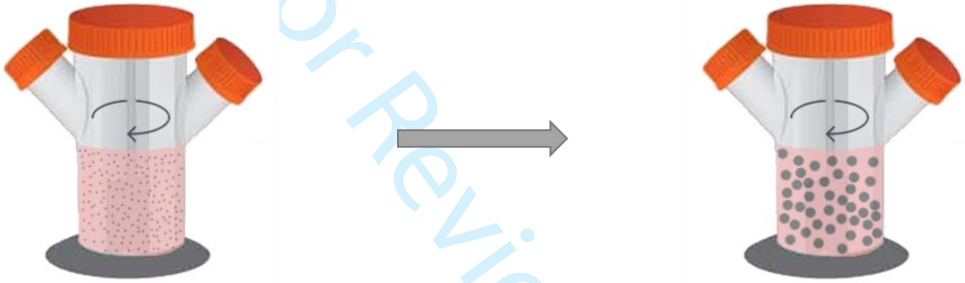

Figure 1: Methods for generation of 3D microtissues. (a) Hanging drop is the first method of generating and maintaining 3D structures in culture. (b) 3D micromoulds have been introduced to the field to overcome both culture media restriction of the hanging drop method and size heterogeneity of spheroids formed in liquid overlay methodology. (c) The use of spinner flask is one of the more advanced methodologies, which is developed for large-scale production and maintenance of 3D microtissues.

As it is difficult to robustly generate 3D microtissues from more than one cell type, several active techniques have been developed to overcome this problem. Active methodologies use additional physical stimuli such as ultrasound traps, electric fields, magnetic forces, or the strong affinity between avidin and biotin to generate multicellular heterospheroids (20-23).

3D organoid formation

Derivation of reconstituted collagen from rat tail (24), discovery of fibronectin (25, 26), isolation of a matrix from chondrosarcoma murine cells (27) and characterisation of

laminin (28) have set the building blocks for subsequent progress in the field of 3D cell culture. In 1989, Barcellos-Hoff and colleagues reported the functional differentiation and alveolar morphogenesis of primary mammary cultures on a reconstituted basement membrane matrix derived from Engelbreth-Holm-Swarm murine tumour, today known as Matrigel™ (29). However, it took nearly two decades to widely utilise the self-organising capacity of cells cultured in this laminin-rich ECM to form 3D organ-like structures known as organoids (30). The generation of organoids has made significant impact and led to the establishment of organoid culture from various tissues (**Fig. 2**), which will be discussed in more details here.

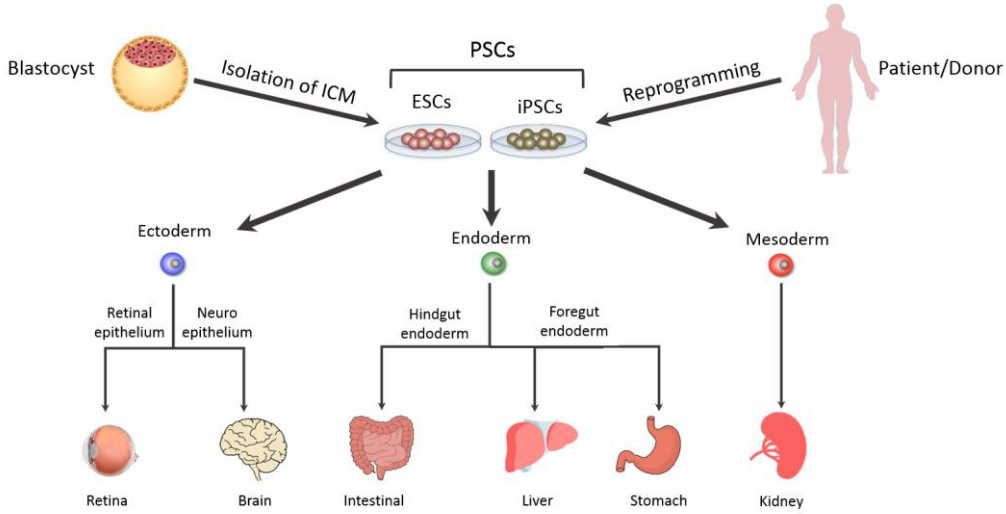

Figure 2: Schematic representation of some of the organoids generated from PSCs. Embryonic stem cells (ESCs) are generated following expansion of cells isolated from the inner cell mass of an embryo at the blastocyst stage while iPSCs can be generated from somatic cells following reprogramming by key master regulators known as Yamanaka factors. Organoids of various tissues have been generated following treatment of Matrigel™-embedded PSCs by cocktails of various growth factors. They can also be generated following isolation and culture of specific populations of progenitor cells, which maintain homeostasis of tissues during adulthood such as cells expressing Leucine-rich repeat-containing G-protein coupled receprot 5 (LGR5).

An insight into 3D organoid cultures

The production of 3D organoid-based culture systems from multiple organs has received considerable attention over the last ten years (31). The term ''organoid'' is defined as

self-organising 3D structures that are cultured *in vitro* while embedded in an ECM. These 3D structures closely resemble their organ of origin (32). Organoids can be derived from various cell sources such as primary tissue, cell lines, adult stem cells (ACS), and pluripotent stem cells (PSCs) (33). Organoids from human PSCs (hPSCs) are great tools to enhance our knowledge of human embryonic development while ASC-derived organoids can closely mimic the *in vivo* stem cell niche and can be considered as useful tools to enhance our understanding of the underlying mechanisms involved in tissue regeneration following injury.

Organoids derived from the intestine and colon

The epithelium of intestine is derived from the definitive endoderm (DE) during embryonic development (34). In a pioneering work, Ootani et al. developed an air-liquid interface model by culturing fragments of intestine, which contained mesenchymal and epithelial cells from neonatal mice. In this model, cyst-like structures were formed in a medium supplemented with foetal bovine serum (FBS). Interestingly, these cyst-like structures were composed of all major cell types of the adult mouse intestine and could be maintained for over one year in culture (35).

Later on, Hans Clevers' research group proposed an alternative technique that aided the formation of epithelial organoids (mini-guts) from single $Lgr5^+$ stem cells. The LGR5 protein is produced by small population of stem cells residing in a variety of adult organs including intestine, stomach, kidney, and skin (36). By using a specialised cell culture medium and the support of Matrigel™ as an ECM, the stem cell niche of the crypt was mimicked and enabled long-term survival of $LGR5^+$ cells (37). These "organoids" were composed of a central lumen surrounded by outgrowths or "buds", which resemble the intestinal crypts and make them distinctive from the cystic structures previously described by Ootani and co-workers (37). In this model, self-renewal of the stem cell population relied on $LGR5^+$ stem cells, which terminally differentiated into enterocytes, and enteroendocrine or goblet cells. This methodological advancement played a key role in mimicking near-physiological conditions of *in vivo* mouse models whilst having an easy-to-maintain *in vitro* culture system (37). Due to the low level of Lgr5 expression, other research groups have investigated other stem cells markers such as CD24 (38), EphB2 (39) and CD166+/GRP78

(40) to generate intestinal organoids. In addition, a step-wise protocol was developed to generate intestinal organoids from hPSCs using Activin A to induce initial transition into DE. Then WNT3A and BMP4 were used to promote hindgut and intestinal specification (41).

Liver organoids

During embryonic development and early hepatogenesis, progenitor cells migrate from the foregut endoderm to form a very dense and vascularised 'liver buds'. The key cross-signalling pathways between mesenchymal, endodermal epithelial and endothelial progenitors have been studied extensively using these to better understand human liver development. In an attempt to recapitulate liver development, 3D aggregates were formed by culturing human PSC-derived hepatocytes with mesenchymal stem cells and endothelial cells on a Matrigel™-coated plate. It was reported that these liver aggregates contained blood vessels and following transplantation into mice become connected to the host vessels within 48 hours. The functional activity of the liver as determined by protein production and drug metabolism activity was significantly increased over time. Further, the recipient mice were recovered from drug-induced liver failure following liver bud transplantation (42).

Hepatocytes and bile duct cells are the two major cell types of the liver, which have extremely slow turn-over in comparison with the small intestine and colon. In the healthy adult mouse liver, Lgr5 is not expressed at high levels. However, upon tissue damage or injury, small $Lgr5^+$ cells located near bile ducts with high level Wnt signalling. It has been reported that following tissue injury, hepatocytes and bile duct cells are generated *in vivo*. With slight alteration, the single $Lgr5^+$ cells could be clonally expanded as organoids by inhibiting notch signalling pathway and differentiation into functional hepatocytes (43). In a similar study, culture conditions were optimised for the long-term expansion of human liver progenitor cells. Similarly, long-term expanded liver organoids remained genetically stable and were transplanted into recipient mice to provide liver support (44).

Pancreatic organoids

The adult pancreas is composed of several different cell types such as exocrine/acinar and endocrine cells with a very slow turn-over. Similar to the liver, under normal

physiological conditions, WNT signalling pathway is not active and the *Lgr5* gene is not highly expressed in the pancreas. Upon tissue injury, the WNT signalling is activated while pancreatic ducts regenerate through proliferation of $Lgr5^+$ cells. In a similar setting to the mini-gut culture condition, clonal pancreas organoids were differentiated and successfully transplanted *in vivo* (45). In an elegant study, Boj and colleagues established organoid models from both normal and neoplastic murine and human pancreatic tissues. Interestingly, these organoids exhibited ductal- and disease stage-specific characteristics and recapitulated tumour progression following *in vivo* transplantation (46).

Lung organoids

The lung is derived from $Nkx2\text{-}1^+$ progenitor cells, which are generated in the ventral foregut endoderm region during embryonic development. In a pioneering work, a cytokeratins 5 *(Krt5)-CreER*^{T2} transgenic mouse model was used to trace and characterise basal cells which act as progenitors to generate differentiated cells during postnatal growth and repair. Following identification of ITGA6 and NGFR as two specific cell surface markers, an organoid culture was established to generate both mouse and human luminal cells including differentiated ciliated cells (47). The generation of lung organoids from PSCs have also been investigated. In an early attempt, induction of PSCs toward the endodermal fate was achieved following Activin A induction and TFG-β/BMP inhibition and subsequent combinatorial induction of BMP and FGF signalling to generate lung progenitors, which can recapitulate the early embryonic development of the lung (48). More recently, an efficient protocol was developed to generate most cell types of the respiratory system including basal, goblet, Clara, ciliated, type I and type II alveolar epithelial cells capable of performing specific functions such as surfactant protein-B uptake and stimulated surfactant release (49).

Stomach Organoids

During embryogenesis, the stomach derives from the posterior foregut. Stomach organoids have been generated from both ASCs and PSCs. D'amour and colleagues proposed a method for the efficient derivation of DE from hESCs. It was reported that in the presence of Activin A and low serum, up to 80% of the cells were differentiated into DE cells. It was also suggested that the process of differentiation into DE requires

epithelial-to-mesenchymal transition (EMT) (50). Later, it was shown that DE can be derived from PSCs with only Activin A following temporal manipulation of retinoic acid, FGF, WNT, BMP, and EGF signalling pathways to generate 3D human gastric organoids (51). In addition, gastric organoids can be generated from single $Lgr5^+$ cells that reside at the base of pyloric glands of the adult mouse stomach. Importantly, generated organoids closely recapitulate mature pyloric epithelium and can be expanded and maintained for an extended period in culture (52). Moreover, at the base of the gastric corpus there are specialised chief cells called Troy cells. Upon exposure to damage, these cells undergo dedifferentiation to become multipotent epithelial stem cells *in vivo*. Using this knowledge, gastric organoids were generated by culturing $Troy^+$ chief cells, which contains various cell types of corpus glands (53).

Brain organoids

During embryonic development, neural ectoderm forms the central nervous system (CNS), initially through formation of the neural plate, which subsequently forms the neural tube via folding and fusion. Similar to other organs, morphogenic gradients in the tube establish a dorsal-ventral and a rostral-caudal axis. Neurons are the major cell types of the CNS and they are generated from neural stem cells (NSC), which located near the ventricles (54, 55).

In ESC culture, spontaneous neural differentiation can be achieved following inhibition of signalling pathways such as BMP, Nodal, and WNTs. This process is very similar to the neural-default mechanism of ESCs. Based on this knowledge, Sasai and colleagues developed SFEBq: serum-free floating culture of embryoid body (EB)-like aggregates with quick re-aggregation (56). In this culture setting, ESCs were isolated from the growth-factor free 2D cultures. The cells were then re-aggregated in 96-well non-adhesive culture plates. The cells were maintained in serum-free medium containing no or a very low levels of growth factors for 7 days, after which they were transferred into adhesion plates. Following formation of the lumen, ESCs polarise and differentiate to generate polarised a neuroectoderm-like epithelium. It was further concluded that under certain conditions, the embryonic spatial and temporal events can be recapitulated *in vitro,* which can in turn lead to the generation of neural structures in the brain (56).

In another study, cerebral organoids were generated and called “mini-brains” as several regions of the brain were represented in each organoid. Very similar to the previous study, the floating EBs were cultured in absence of growth factors to derive specific brain region identity. Further, aggregates were embedded in a laminin-rich ECM. With this technique, large neuroepithelial buds were formed representing different brain regions. Interestingly, it was reported that brain regions such as retina, ventral forebrain, midbrain-hind-brain boundary, and dorsal cortex were observed in these cultures (55).

Retinal organoids

Embryonic development of the retina occurs through lateral evagination of the diencephalon, which in turn forms pseudostratified neuro-epithelial known as optic vesicles (OVs). Later, sensory neural retina (NR) is derived from the distal portion of the OVs, while the proximal portion gives rise to retinal pigment epithelium (RPE). Following invagination of OVs at their distal portion, a bi-layered optic cup (OC) is formed with the RPE and NR as its outer and inner walls, respectively. The NR progenitor cells give rise to photoreceptors (rods and cones), ganglion cells, and all supportive cell types (57). Pioneering work in chick embryos that demonstrated the retinal capacity to form different cell types in the distinct laminated structure of retina paved the way for development of PSC-derived retinal organoids (58).

Following an initial studies that demonstrated the successful formation of retinal epithelium from 3D floating mESC-derived EB-like aggregates in a low-serum medium (59), retinal organoids were generated from self-organising hESCs forming a multi-layered tissue containing both rod and cone photoreceptors. Remarkably, retinal organoids formed from hESCs were much larger in size than organoids derived from mESCs, potentially reflecting the species-specific differences (60).

Other organs

More recently, organoids from other organs such as the prostate (61), fallopian tube (62), mammary gland (63, 64), taste buds (65), salivary glands (66, 67), and oesophagus (68) have all been developed.

The basal and luminal cells are two major cell types that form the pseudostratified epithelium of the prostate. In 2014, a mini-gut-based culture method was developed to support the long-term expansion of primary mouse and human prostate organoids. The structure of these 3D organoids consisted of mature and differentiated basal and luminal cells. It was also reported that luminal cell-derived organoids closely resembled prostate glands. Luminal cell induction depends on WNT or R-spondin activation to some extent and subsequently this will form prostate-like pseudostratified organoid structures (61). Furthermore, an alternative culture system was established to derive prostate organoids using Matrigel™, EGF, and androgen supplementation independently (69).

The fallopian tube is an anatomically simple organ, which is composed of columnar epithelium. Secretory cells produce tubular fluid and ciliated cells support the transfer of gametes within the tube. Self-renewal capacity of the epithelium is of utmost importance due to the monthly cyclical hormonal fluctuations. In 2015, long-term 3D organoid culture of the human fallopian tube was established following adaptation of mini-gut culture protocols. The resulting clonal organoids were composed of ciliated and secretory cells, which provide the opportunity to study human fallopian tube epithelium in more details (62).

Applications of 3D Organoids

Organoids can be exploited for various applications such as disease modelling, drug toxicity testing, organoid biobanking, personalised therapy, and host-pathogen interaction studies. In addition, organoids are a useful tool to perform omics analysis (transcriptomics, proteomics, epigenomics and metabolomics) of healthy and diseased tissues to gain a better understanding of mechanisms underlying pathological conditions (70). Some of these applications are discusses below in further detail.

Disease modelling

Although several animal models have been generated to recapitulate clinical characteristics of human monogenic disorders following introduction of single-gene mutations, introduction of such a mutation does not guarantee the recapitulation of the clinical features of these disorders in recipient animals. However, organoids generated from patient-specific iPSC lines can recapitulate the clinical features of various

monogenic disorders and can be used as *in vitro* models to further study these disorders.

A clear example is an early attempt to generate an *in vitro* model of cystic fibrosis (CF) using patient-derived tissue fragments (71). CF is an autosomal recessive genetic disorder caused by a mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene affecting multiple organs including the lung, intestine, liver, pancreas, and reproductive tract (72). Dekkers *et al.* initially developed an organoid-based assay, whereby forskolin promoted a rapid swelling of wild type-derived organoids from mouse and human intestinal samples through activation of c-AMP. They further concluded that the drug-induced swelling was significantly reduced in mice carrying the F508del mutation in the CFTR disease model. With the development of this advanced methodology, it was suggested that this is a promising tool to study gene therapy models to correct CFTR mutations (71). In a follow up study, the same assay was used to assess the potential of CRISPR/CAS9 technology to correct the CFTR F508del allele. Interestingly, organoids with the correct set of alleles regained the ability to swell upon exposure to forskolin. These studies concurrently demonstrated a proof-of-concept for gene replacement therapy for future clinical translation (73). More recently, *in vitro* organoid models of other monogenic disorders such as Alagille syndrome (74), and Retinitis Pigmentosa (75) have been generated, which is reviewed more extensively elsewhere (76).

Cell-based therapies

Despite advanced in therapeutic regimens, there are various inherited, degenerative and, chronic disorders that have remained incurable through conventional approaches. Stem cell-based therapies have the potential to alleviate symptoms or possibly cure these conditions by replacing damaged or lost cells. The ability to generate organ-like structures, which contain representative cell populations of the desired organs has made organoid culture a powerful tool to obtain various progenitor cells for cell-based therapies.

In an early attempt, Yui and co-workers prepared a large batch of organoids, which originally derived from single $Lgr5^{+}$ colon stem cells to study long-term genetic stability of the organoids. These organoids were transplanted per annum into multiple mice

suffering from experimental colitis. They further confirmed that organoids were readily integrated and acted as functional epithelial patches, which could not be easily distinguished from the host epithelium (77). In another elegant study, improvement of vision impairment was successfully demonstrated following transplantation of functional rod photoreceptors in adult Gnat1$^{-/-}$ mice, which lack rod function as a model of congenital stationary night blindness (78). Therefore, generation of transplantation-competent photoreceptor precursors from hPSCs have been investigated to treat blindness (79-82).

In addition, multi-lineage approaches have been developed to generate composite organoids for the liver, lung, intestine, heart, kidney, and brain (42, 83). Despite promising outcomes in the preliminary studies, clinical translation of hPSC-derived organoids faces several major challenges including reliance of current protocols on undefined and animal-derived ingredients that need to be resolved to facilitate their clinical applications.

Drug screening, organ-on-chips, and personalised medicine

High attrition rate is the biggest challenge facing the pharmaceutical industry. Lack of suitable preclinical models to accurately predict efficacy and toxicity of novel lead compounds has been considered as one of the major contributors. To improve productivity and predictability, 2D cell-based screenings have been used as a convenient means to evaluate novel therapeutic candidates. However, the emerging evidences has revealed poor predictability of 2D screening platforms for certain diseases such as cancers (84). In addition, predictability of preclinical animal models has been a matter of debate due to considerable interspecies differences in disease phenotypes and reactions to drugs (85-87). Lack of predictability and growing ethical concerns regarding the use of laboratory animals have encouraged exploration of new avenues to develop novel screening platforms to mitigate the high attrition rate.

To overcome these issues, various mono- and co-culture 3D systems have been developed for oncology research and drug screening. Nutrients, oxygen, metabolites, and soluble factors induce the formation of a heterogeneous population of cells within 3D microtissues to mimic tumour microenvironments more closely than monolayer cultures (88).

Despite various practical challenges, 3D drug screening platforms have grown in popularity and both tumour and healthy organoids of various tissues have been generated from patients' biopsies and ASCs or PSCs. In a pioneering work, Wong et al. demonstrated the usefulness of *in vitro* organoid models for the screening of lead compounds following treatment of patient-derived organoids with a novel small molecule to correct for a common CF-processing mutation that resulted in enhanced membrane localisation of mature CFTR protein (89).

Considering heterogeneity of tumour pathophysiology, patient-derived organoids have proven to be a useful tool for cancer drug discovery. The heterogeneous response of neoplastic tumours to anti-cancer treatment was demonstrated following screening of 83 authorised and experimental anti-cancer agents on tumour organoids derived from resected colorectal tissues obtained from 20 patients (90). Similarly, organoids from three major subtypes of liver cancers were propagated and used for drug screening. Interestingly, liver cancer-derived organoids preserved gene expression, genomic landscape, and metastatic properties of the original tumours even after long-term *in vitro* expansion. In addition, SCH772984 (an extracellular signal-regulated kinase (ERK) inhibitor) was identified as a potential therapeutic compound for primary liver cancer (91).

Above mentioned studies reiterate the importance of patient-specific organoids to identify an appropriate anti-tumour regimen for the efficient treatment of neoplastic disorders. To this end, organoid biobanks have been established from patient tumours as a valuable tool for drug screening and personalised medicine (90, 92, 93).

3D organoids have also been used in conjunction with microfluidic devices, known as organ-on-chips, as a powerful tool for drug screening. Although organ-on-chips are designed to represent functional complexity of a particular organ such as the intestine (94) and liver (95), recent efforts have been focused on the development of more sophisticated platforms by interconnecting several organ-on-chips (96). Development of such platforms can substitute mandatory preclinical studies in animal models to increase the success rate and improve productivity of drug screening while addressing growing ethical concerns regarding the use of animal models for drug screening.

Modelling infectious diseases to mimic complex interaction between the host and pathogens

The Zika Virus (ZIKV) is a flavivirus, which was isolated from a rhesus monkey in the Zika region of Uganda in 1947 and can be transmitted by *Aedes* species mosquitoes (97). Following entry to the human body, ZIKV binds to innate immune Toll-like receptor 3 (TRL3), which leads to the activation of genes causing disregulation of neurogenesis, which is a common side effect seen following ZIKV infection. Using hESC-derived cerebral organoids, it was demonstrated that TLR3 inhibition reduced the phenotypic effects of ZIKV infection (97). Other studies also suggested that the mechanism of action of this lethal virus is concerned with TRL3-mediated apoptosis, hence cell death of neural stem cells (NSC) and impaired development in humans (98, 99). These experiments also demonstrated that microcephaly (i.e; a low level of NSC proliferation and more cell death) can be observed as a side effect of ZIKV infection in organoids (98, 100). Based on this knowledge, another research group employed a unique miniaturised spinning bioreactor system to grow forebrain-specific organoids derived from hiPSCs to be used as a major platform for high-throughput drug screening (101).

Techniques for the introduction of microorganisms into organoids

Organoids are dense 3D structures, which are composed of apical and basal membranes as two main compartments. The apical side of the epithelium is toward the lumen (inside) of the organoids and the basal membrane appears on the outside. Microorganisms tend to target the apical membrane *in vivo*. Therefore, recapitulating the exact interactions between the host and the microbes are crucial. Hence, three independent strategies have been developed to reproduce host versus pathogen interactions (70).

I. Infection of dissociated spheroids before forming 3D organoids

In this technique, organoids are forced to undergo mechanical shear stress or enzymatic digestion to become single-cell suspension to expose the apical side. Following infection of dissociated cells, the infected cells will be seeded in a 3D matrix to form 3D organoids within a few days. This method was employed to study gene expression manipulations using a specific lentiviral system (102) and can be used to model different infectious disease models (97, 103, 104).

II. Microinjection of viruses or bacteria into the lumen side of organoids

This technique was previously developed to inject ESCs into mice to study genetics. With slight modifications, microorganisms can be injected directly into the organoid's lumen (105, 106). As the organoids remain intact and no dissociation occurs, the necessary interaction between the host and pathogens can be easily detected and monitored. Although this method seems promising, there are some limitations including the availability of a microinjector device and precise quantification of delivered pathogens can be difficult due to the size variation of organoids in culture (70).

III. 2D culture derived organoids and interaction with microorganisms

3D organoids can be dissociated and seeded onto an ECM such as Matrigel™ or collagen-coated plates. The cells will expand in 2D and the apical surface will be exposed on the surface, therefore, when microorganisms are added to the dish, the host-microbe interaction proceeds. With this technique, microbes can be quantified, however; it does not resemble the *in vivo* 3D setting (107).

Future Directions

The ability to generate organ-specific organoids using hPSCs or tissue-specific progenitor cells alongside the development of cancer organoids has made organoid technology a powerful tool to study various biological aspects including organ development, tissue morphogenesis, modelling diseases *in vitro*, and testing the efficacy and toxicity of therapeutic compounds (41, 43, 44, 51, 55, 71, 91, 108-112). The advancement in microfabrication and microfluidic technology can set the stage for the development of new devices to enable high-throughput screening and biosensing, which subsequently would expand organoid application as a tool for drug toxicity screening of novel compounds (113).

To achieve the full potential of 3D organoids, it is important to overcome limitations associated with current methodologies, particularly phenotypic immaturity of derived cells. For instance, suboptimal expression of hepocyte-specific CYP450 enzymes and low levels of albumin secretion were reported in liver organoids compared to primary hepatocytes, which restricts their downstream industrial and clinical applications (114). In addition, Matrigel™, as an undefined animal product, has been an indispensable element of 3D organoid methodologies that would undermine their therapeutic value.

Therefore, it is important to develop new methodologies to establish GMP-ready protocols for the generation of 3D microtissues by using xeno-free and well-defined matrices to facilitate their potential clinical applications.

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