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Three-dimensional cell culture: from evolution to revolution

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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 along specific lineages have facilitated the development of high-fidelity modelling of several tissues *in vitro*. Many of the novel approaches have employed self-organizing three-dimensional (3D) culturing of organoids, which offer several advantages over conventional two-dimensional platforms. Organoid technologies hold great promise 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 three-dimensional organoids. We also highlight some of the current limitations of these systems and discuss potential avenues to further benefit biological research using three-dimensional modelling technologies.

This article is part of the theme issue 'Designer human tissue: coming to a lab near you'.

1. Introduction

The isolation and maintenance of mammalian cells have significantly advanced scientific research into cellular processes and mechanisms of disease, including 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 monolayer culture [5].

Since then, culturing cells in two-dimensional format has remained the predominant methodology of *in vitro* cell growth and expansion. However, the two-dimensional platforms do not effectively recapitulate the spatial requirements that are essential for the organization and cellular interactions that 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 two-dimensional *in vitro* versus *in vivo* research.

2. Historical background

To overcome two-dimensional platform limitations, efforts have led to the development of novel approaches to recreate a more physiologically relevant environment in the form of three-dimensional cell culture [1]. To successfully construct and maintain a three-dimensional structure, much research has been devoted to the development of synthetic or natural polymeric three-dimensional scaffolds to facilitate cell growth. These efforts have resulted in the fabrication and characterization of several non-degradable or biodegradable synthetic polymers, such as poly-lactic acid, poly-glycolic acid, poly-lactic-*co*-glycolic acid and

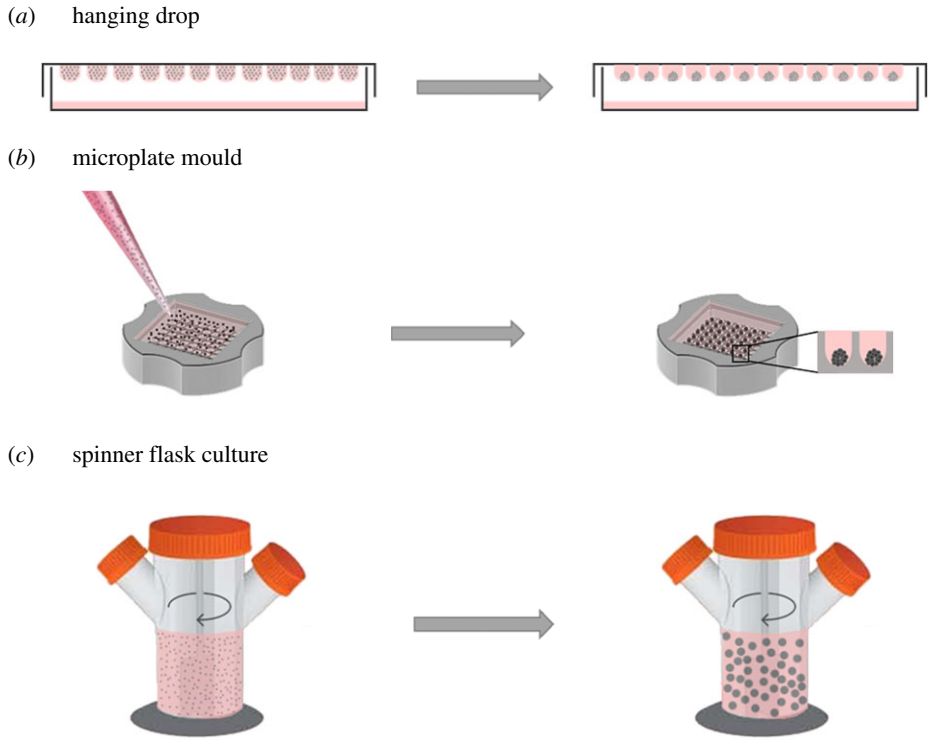

Figure 1. Methods for generation of three-dimensional microtissues. (*a*) Hanging-drop was the first method of generating and maintaining three-dimensional structures in culture. (*b*) Three-dimensional micromoulds have been introduced to the field to overcome both culture media restriction of the hanging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

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 cellularized blocks and layer-by-layer assembly (also known as three-dimensional printing) have been developed [8]. In this review, we focus on scaffold-free methods to culture cells in three-dimensions and the generation of organoids by embedding cells in semi-solidified extracellular matrices (ECM) in contrast to the use of polymeric scaffolds and three-dimensional printing, which have been reviewed extensively elsewhere [6,9,10].

3. Techniques to generate scaffold-free three-dimensional cellular aggregates

In general, scaffold-free approaches rely on cell–cell interaction and can be categorized 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 three-dimensional aggregates robustly and consistently (figure 1).

Hanging-drop was the first technique to generate three-dimensional structures, by culturing suspended droplets of the desired cell line(s) to force aggregation (figure 1*a*). 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 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 twentieth century. Although three-dimensional 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 three-dimensional 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, the 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 (figure 1*b*) and patterned microplates have more recently been used [19].

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

As it is difficult to robustly generate three-dimensional 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].

(a) Three-dimensional organoid formation

Derivation of reconstituted collagen from rat tail [24], the discovery of fibronectin [25,26], isolation of a matrix from

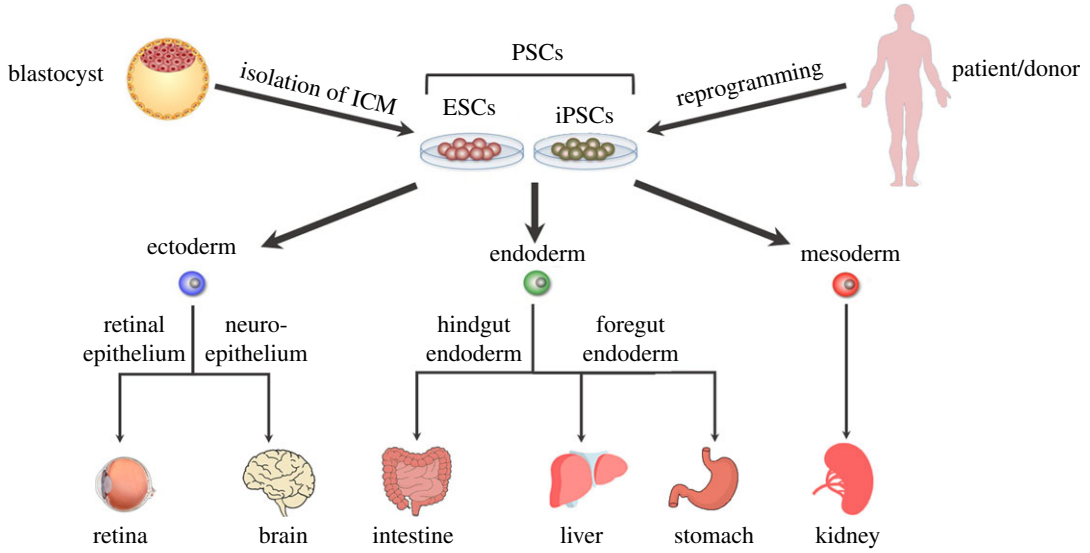

Figure 2. Schematic of some of the organoids generated from pluripotent stem cells (PSCs). Embryonic stem cells (ESCs) are generated following the expansion of cells isolated from the inner cell mass of an embryo at the blastocyst stage while iPSCs can be generated from somatic cells following reprogramming by key master regulators known as Yamanaka factors. Organoids of various tissues have been generated following treatment of Matrigel™-embedded PSCs with 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 receptor 5 (LGR5). (Online version in colour.)

chondrosarcoma murine cells [27] and characterization of laminin [28] have set the building blocks for subsequent progress in the field of three-dimensional 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 use the self-organizing capacity of cells cultured in this laminin-rich ECM to form three-dimensional 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 (figure 2), which will be discussed in more detail here.

4. An insight into three-dimensional organoid cultures

The production of three-dimensional organoid-based culture systems from multiple organs has received considerable attention over the last 10 years [31]. The term 'organoid' is defined as self-organizing three-dimensional structures that are cultured *in vitro* while embedded in an ECM. These three-dimensional 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 (ACSs) 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.

(a) 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 that contained mesenchymal and epithelial cells from neonatal mice. In this model, cyst-like structures were formed in a medium supplemented with fetal bovine serum. Interestingly, these cyst-like structures were composed of all major cell types of the adult mouse intestine and could be maintained for over 1 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 a small population of stem cells residing in a variety of adult organs including intestine, stomach, kidney and skin [36]. By using a specialized 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 [35]. 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 while having an easy-to-maintain *in vitro* culture system [37]. Owing 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].

(b) Liver organoids

During embryonic development and early hepatogenesis, progenitor cells migrate from the foregut endoderm to form

very dense and vascularized '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, three-dimensional 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 h. The functional activity of the liver as determined by protein production and drug metabolism activity was significantly increased over time. Furthermore, 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 in 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 wingless (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 the notch signalling pathway and differentiation into functional hepatocytes [43]. In a similar study, culture conditions were optimized 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].

(c) 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, the Wnt signalling pathway is not active and the *Lgr5* gene is not highly expressed in the pancreas. Upon tissue injury, 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].

(d) Lung organoids

The lung is derived from Nk2 homeobox1$^+$ (Nkx2.1$^+$) 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 characterize basal cells which act as progenitors to generate differentiated cells during postnatal growth and repair. Following identification of integrin subunit alpha 6 (ITGA6) and nerve growth factor receptor (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]. In order to model lung development *in vitro*, the generation of lung organoids from PSCs has also been investigated. In an early attempt, induction of PSCs towards the endodermal fate was achieved following activin A induction and TFG-β/BMP inhibition and subsequent combinatorial induction of BMP and fibroblast growth factor (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].

(e) 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 [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 epithelial growth factor (EGF) signalling pathways to generate three-dimensional 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 specialized 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 using a previously established protocol that allows long-term expansion of single *Lgr5*$^+$ pyloric stem cells [53].

(f) Brain organoids

During embryonic development, neural ectoderm forms the central nervous system (CNS), initially through the 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 (NSCs), which are 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 WNT. 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 growth-factor-free two-dimensional 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 very low levels of growth factors for 7 days, after which they were transferred into adhesion plates. Following formation of the lumen, ESCs polarize and differentiate to generate polarized 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 that were 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 the absence of growth factors to derive specific brain region identity. Further, aggregates were embedded in a laminin-rich ECM. With this technique, large neuro-epithelial 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].

(g) Retinal organoids

Embryonic development of the retina occurs through lateral evagination of the diencephalon, which in turn forms pseudostratified neuro-epithelium known as optic vesicle (OV). 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 the distal portion, a bi-layered optic cup is formed with the RPE and NR at 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 the development of PSC-derived retinal organoids [58].

Following initial studies that demonstrated the successful formation of retinal epithelium from three-dimensional floating mouse ESC-derived EB-like aggregates in a low-serum medium [59], retinal organoids were generated from self-organizing human ESCs forming a multi-layered tissue containing both rod and cone photoreceptors. Remarkably, retinal organoids formed from human ESCs were much larger in size than organoids derived from mouse ESCs, potentially reflecting species-specific differences [60].

(h) 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 three-dimensional 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^{TM}, 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 owing to the monthly cyclical hormonal fluctuations. In 2015, the long-term three-dimensional 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].

5. Applications of three-dimensional organoids

Organoids can be exploited for various applications such as disease modelling, drug toxicity testing, organoid biobanking, personalized 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 discussed below in further detail.

(a) Disease modelling

Although several animal models have been generated to recapitulate clinical characteristics of human monogenic disorders following the introduction of single-gene mutations, the 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 cyclic 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 are reviewed more extensively elsewhere [76].

(b) Cell-based therapies

Despite advances 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 organoids that 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 has 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 on current protocols on undefined and animal-derived ingredients, that need to be resolved to facilitate their clinical applications.

(c) Drug screening, organ-on-chips and personalized 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 to be one of the major contributors. To improve productivity and predictability, two-dimensional cell-based screenings have been used as a convenient means to evaluate novel therapeutic candidates. However, the emerging evidence has revealed poor predictability of two-dimensional screening platforms for certain diseases, such as cancers [84]. In addition, the predictability of preclinical animal models has been a matter of debate owing 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 three-dimensional 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 three-dimensional microtissues to mimic tumour microenvironments more closely than monolayer cultures [88].

Despite various practical challenges, three-dimensional 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 localization 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 authorized 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 inhibitor) was identified as a potential therapeutic compound for primary liver cancer [91]. More recently, organoids were derived from a large number of patients enrolled in four prospective phases 1 and 2 clinical trials. While notable morphological similarities were observed between patient-derived organoids and the patient biopsies from which they were originally derived, the data from a screening of anti-cancer agents suggested that patient-derived organoids can recapitulate patient responses in the clinic [92]. The 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 personalized medicine [90,93,94].

Three-dimensional 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 the functional complexity of a particular organ such as the intestine [95] or liver [96], recent efforts have been focused on the development of more sophisticated platforms by interconnecting several organ-on-chips [97]. Development of such platforms can substitute for mandatory preclinical studies in animal models to increase the success rate and improve the productivity of drug screening while addressing growing ethical concerns regarding the use of animal models for drug screening.

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

The Zika virus (ZIKV) is a flavivirus that was isolated from a rhesus monkey in the Zika region of Uganda in 1947 and can be transmitted by *Aedes* species mosquitoes [98]. 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 [98]. Other studies also suggested that the mechanism of action of this lethal virus is concerned with TRL3-mediated apoptosis, hence cell death of NSC and impaired development in humans [99,100]. 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 [99,101]. Based on this

knowledge, another research group employed a unique miniaturized spinning bioreactor system to grow forebrain-specific organoids derived from hiPSCs to be used as a major platform for high-throughput drug screening [102].

(e) Techniques for the introduction of microorganisms into organoids

Organoids are dense three-dimensional structures that are composed of apical and basal membranes as two main compartments. The apical side of the epithelium is towards 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 is crucial. Hence, three independent strategies have been developed to reproduce host–pathogen interactions [70].

(i) Infection of dissociated spheroids before forming three-dimensional organoids

In this technique, organoids are forced to undergo mechanical shear stress or enzymatic digestion to become single-cell suspensions to expose the apical side. Following infection of dissociated cells, the infected cells are seeded in a three-dimensional matrix to form three-dimensional organoids within a few days. This method was employed to study gene expression manipulations using a specific lentiviral system [103] and can be used to model different infectious disease models [98,104,105].

(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 [106,107]. 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) Two-dimensional culture-derived organoids and interaction with microorganisms

Three-dimensional organoids can be dissociated and seeded onto an ECM such as Matrigel™ or collagen-coated plates. The cells will expand in two-dimensions 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* three-dimensional setting [108].

6. 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 [41,43,44,51,55,71,91,109–113]. 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 [114].

To achieve the full potential of three-dimensional 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 with primary hepatocytes, which restricts their downstream industrial and clinical applications [115]. In addition, Matrigel™, as an undefined animal product, has been an indispensable element of three-dimensional organoid methodologies that would undermine their therapeutic value. Therefore, it is important to develop new methodologies to establish protocols that are compatible with current good manufacturing practice for the generation of three-dimensional microtissues by using xeno-free and well-defined matrices to facilitate their potential clinical applications.

Data accessibility. This article has no additional data.

Competing interests. We declare we have no competing interests.

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