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Biomaterials and Culture Systems for Development of Organoid and Organ-on-a-chip Models

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Abstract

The development of novel 3D tissue culture systems has enabled the *in vitro* study of *in vivo* processes, thereby overcoming many of the limitations of previous 2D tissue culture systems. Advances in biomaterials, including the discovery of novel synthetic polymers has allowed for the generation of physiologically relevant *in vitro* 3D culture models. A large number of 3D culture systems, aided by novel organ-on-a-chip and bioreactor technologies have been developed to improve reproducibility and scalability of *in vitro* organ models. The discovery of induced pluripotent stem cells (iPSCs) and the increasing number of protocols to generate iPSC-derived cell types has allowed for the generation of novel 3D models with minimal ethical limitations. The production of iPSC-derived 3D cultures has revolutionized the field of developmental biology and in particular, the study of fetal brain development. Furthermore, physiologically relevant 3D cultures generated from PSCs or adult stem cells (ASCs) have greatly advanced *in vitro* disease modelling and drug discovery. This review focuses on advances in 3D culture systems over the past years to model fetal development, disease pathology and support drug discovery *in vitro*, with a specific focus on the enabling role of biomaterials.

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1. Introduction

Historically, *in vitro* tissue culture has been conducted primarily in a 2D setting with at most a co-culture of two to three different cell types. Although informative and easily accessible, this method provides an oversimplified understanding of what is naturally a multiplex system. Models cultured in static conditions on hard plastic surfaces cannot fully recapitulate the chemical and mechanical cues to promote similar cell-cell and cell-ECM interactions *in vivo*. Studying a complex system such as the human body in discrete sections may neglect biophysical and 3D matrix related factors that will undoubtedly impact the crosstalk between different cell types or alter critical signaling pathways. Additionally, to run such experiments, only a select number of variables could be simultaneously compared, requiring a well-defined and attributive experimental hypothesis. Regardless, due to the conventional methods for 2D culture and the breadth of research available for benchmarking, little has been done to expand the boundaries of *in vitro* modelling.

Given the challenge in translating research findings from *in vitro* experiments and animal models to clinical trials, many have expressed the need for an intermediate stage where novel findings can be tested against human-specific models. The integration of engineering principles with fundamental biological knowledge has further led to significant developments in 3D culturing techniques, enhancing the ability of researchers to reproduce physiologically relevant environments *in vitro*. This review will provide an overview of commonly used biomaterials and novel three-dimensional culture systems such as organoids and organ-on-a-chip systems, as well as the applications of these technologies in developmental and disease models and high-throughput drug testing. With a vast range of novel biomaterials at our disposal, researchers are rapidly moving away from plastic culture and transitioning into intricate microfabricated 3D models that capture specific tissue functions, as seen in the development of organ-on-a-chip systems[1]. Likewise, the availability of the organ-specific cells has increased tremendously, due to the improved understanding of developmental biology and advancement in stem cell technology. These critical steps allow for successes in 3D tissue development and the creation of functional engineered tissues[2]. Here, we defined organoids as spherical aggregates of cells that occur due to the spontaneous differentiation of either adult or pluripotent stem cells. Organs-on-a-chip are defined as we stated previously, as cell culture methods that have a significant engineering component, such as guided spatial confinement of cells or incorporation of sensors and microfluidic channels[32]. Significant advancements have occurred in models for cardiac, pulmonary, renal as well as hepatic tissues but more recently there has been a vested interest in examining 3D culture techniques for the development of long-term neural models[3–6]. The authors will also address the drawbacks of existing models and propose strategies for enhancing their accuracy.

With the development of more complex 3D models and the ability to customize a system on a patient-specific basis, such models may be better predictors of human physiology than animal models. Additionally, with the increasing accessibility of genetic sequencing tools, these systems will likely be more economical and provide faster results. However, with the present number of competing 3D models, further testing will be necessary to standardize these systems and improve the reproducibility of experimental outcomes. 3D culture

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modelling is indisputably superior to its predecessors but will require a substantial amount of additional investigation prior to its adaptation for widespread clinical use.

2. Sources of Cells for *In Vitro* 3D Culture Models

3D culture models can be defined as the growth and interaction of cells *in vitro* in a three-dimensional space. Although a range of cell types can be grown *in vitro* under 3D conditions, this review focuses on culture models derived from stem and stem-like cells. Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are commonly used to generate 3D tissues *in vitro* through regulation of differentiation cues (Figure 1A). Alternatively, adult stem cells can be isolated from volunteers and grown *in vitro* under conditions that support the formation of 3D tissues (Figure 1B). 3D cancer models generated *in vitro* using cancer cells isolated from patients are referred to as tumour organoids.

A specific sub-set of 3D cell culture are organoids. Organoids are self-organizing *in vitro* organ-like 3D structures that are formed from PSC-derived (Figure 2) or adult stem cell-derived progenitors (Figure 1B) and contain multiple cell types that resemble the *in vivo* organ and retain some physiological function[7]. These systems are most often generated relying on self-aggregation of differentiating cells. However, they can be formed by embedding cells in hydrogels containing polymers or extracellular matrix components[8]. The predominantly used biomaterials for culturing organoids are Matrigel and collagen. Garnier and colleagues enhanced and stabilized culture conditions for human hepatocytes in Matrigel hydrogels[9]. Qian and colleagues have also used Matrigel to culture specific regions of the brain by using embryoid bodies[10]. Furthermore, this study grew brain organoids in spinning bioreactors to generate parts of the forebrain from iPSCs. Synthetic polymers were explored using polyethylene glycol, a synthetic polymer for organoid formation as demonstrated by Blondel and colleagues for mouse and human intestinal modelling[11]. Furthermore, functionalizing polyethylene glycol with a peptide, RGDSP, allowed for enhanced adherence of mouse intestinal stem cells. Considering the range of biomaterials available, organoid cultures are able to replicate various tissue types including brain, heart, kidney, retinal, intestinal and colon among others[12–17]

2.1. Pluripotent Stem Cell (PSC)-derived 3D Culture Models

The most commonly used technique to generate *in vitro* 3D culture models begins with PSCs, either iPSCs or ESCs, that are differentiated into specific cell types of interest using a defined protocol. PSCs can initially be differentiated under 2D or 3D culture conditions and later combined with other differentiated cell types or commercially available cell lines to generate 3D co-cultures. 3D tissue models from all three germ layers – ectoderm, endoderm and mesoderm have been generated using this workflow (Table 1) with cerebral, pancreatic and kidney organoids as examples for each germ layer, respectively[18–20]. These 3D models can be used to study tissue development as well as congenital conditions and developmental defects *in vitro*, ultimately leading to the establishment of more efficacious treatments. The main limitation of PSC-derived 3D models is that the tissues generated resemble embryonic or fetal rather than adult tissues. This raises the question of whether 3D tissues generated *in*

vitro from PSCs will be useful for studying adult diseases or in the development of transplantation therapies, emphasizing the need for bioengineering approaches that can generate mature tissue.

Organoid models can be derived from PSCs by first inducing differentiation into a specific germ layer, followed by further differentiation into lineage-specific progenitors upon aggregation[21]. For example, a model of neural tube formation was generated by culturing mouse ESCs under neural induction conditions to form 3D neuroepithelia, which could be further patterned through the addition of Sonic Hedgehog or retinoic acid[22]. The addition of retinoic acid to these 3D neuroepithelial cultures spontaneously formed a floor plate, which resulted in the generation of various dorsal and ventral neural subtypes. Pancreatic organoids were generated from iPSC-derived pancreatic progenitors, which resulted in mature pancreatic cell types upon prolonged culture, including differentiated β-cells that secreted insulin in response to glucose treatment[23]. Cerebral organoids grown from iPSC-derived neural progenitors have been used to generate 3D tissue to model brain development *in vitro*[18]. However, a major limitation of PSC-derived organoids is the resemblance to fetal organs and the requirement of extended *in vitro* culture to generate more mature fates. For example, PSC-derived cardiac organoids present an immature action potential profile, calcium transients and glucose-dependent metabolic activity similar to the human fetal heart[12,24–26]. Human cerebral organoids also share similar proteomic profiles with fetal brain tissues.[27,28]

2.2. Adult Stem Cell-derived 3D Culture Models

An alternative to PSC-derived 3D culture models is the use of adult stem cells isolated from healthy volunteers or patients (Figure 1B). These adult stem cells, such as hematopoietic stem cells, bone marrow stem cells, adipose-derived stem cells and intestinal stem cells, can be isolated from a patient biopsy, surgical resection or donated healthy and diseased organs, and can serve as cell sources for both normal and tumour organoids[29–31]. These cells can be obtained without ethical limitations related to the use of ESCs and have the potency to differentiate into many cell types *in vitro*. However, they are typically only multipotent and do not have the same differentiation potential as PSCs[32–34]. The advantage of using adult stem cells over PSCs is the ability to generate 3D models of adult tissue *in vitro*, due to the fetal-like phenotypes reported from PSCs differentiation[35–38]. Therefore, this system is useful for studying tissue homeostasis, organ injury and repair, cellular aging and the mechanisms of age-related diseases such as cancer. These cell types are also well suited as a source for autologous transplantation therapies. Some organs for which these models have been developed are shown in Table 2, including the small intestine, colon, liver, pancreas and lung[15,37,39–41]. However, the acquisition of adult stem cells is more invasive, expensive and relies on the presence of stem cell pools within the tissue of interest. Thus, obtaining adult stem cells can be a limiting factor depending on the invasiveness of the surgery required. Another challenge is the longevity of the cultures, since some developed models cannot be cultured for long periods of time. However, this can often be resolved through optimization of the *in vitro* culture conditions.

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As previously described, patient-derived adult stem cells can be used to generate organoids resembling healthy adult organs. Various epithelial organoid models have been generated using this methodology. The first adult stem cell-derived organoid models were generated by embedding isolated intestinal crypts or sorted epithelial cells in Matrigel, which expanded to form intestinal and colonic organoids[15].

A common problem of static 3D cultures, including both spheroids and organoids, is the presence of a necrotic interior due to the nutrient diffusion limit and a lack of vasculature, though this can be partially solved by the use of bioreactors that better circulate nutrients. Further development of organoid culture models through co-culture of additional cell types such as stromal, immune or vascular cells is also required to create models that better mimic the *in vivo* organ[30]. Since organoids are generated by aggregation and self-organization of stem and progenitor cells, this affects the reproducibility of the system. These shortcomings are essential to the downstream applications discussed in this review.

2.3. Tumour Organoids

The growth of patient-derived tumour samples *in vitro* under 3D conditions is referred to as tumour organoids. In these culture systems, cancer cells isolated from patients are grown under optimized growth conditions resulting in a 3D aggregate of tumour cells. Tumour organoids are increasingly being used as models to study tumorigenesis and disease progression *in vitro* and as models for drug discovery and personalized medicine. The benefit of such models over traditional cancer cell lines is that tumour organoids provide a physiologically relevant system that maintains the heterogeneity of the original tumour[42]. Additionally, many tumour organoid models consist of different cell types, allowing the study of niche-tumour interactions *in vitro*. Examples of patient-derived tumour organoid models developed thus far include models of colorectal cancer (CRC), glioblastoma multiforme (GBM), breast and lung cancer[29,43–46]. The previously developed tumour organoid models have shown the benefit of using patient-derived primary cells combined with 3D culture systems to more accurately model the tumour environment compared to traditional cancer cell models. Advancements in these tumour organoid models will allow for improved drug discovery and personalized medicine.

3. Biomaterials Facilitating 3D Tissue Models

A key difference between 2D and 3D tissue models is the use of biomaterials, in which cells reside, in order to better simulate *in vivo* physiological conditions. The development of novel *in vitro 3D* models has largely been possible due to the advancement and discovery of biomaterials. These biomaterials can be designed to form various 3D scaffolds with functional groups, i.e. through photo-polymerization, to achieve the optimal microenvironment for cell growth. As an example, stereolithography technology utilizes UV energy to 3D print scaffolds, thereby creating custom designs to match numerous applications.

Biomaterials are classified as either synthetic, naturally derived or hybrid composites of the former two. Each biomaterial has its own unique properties that must meet specific requirements to be considered for use in 3D tissue modelling. For *in vitro* uses, the

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mechanical and chemical properties of the biomaterials should be tailored for optimal cellular attachment and growth. The recapitulation of tissue-specific architecture is also preferred. For *in vivo* applications, the biomaterial must have a minimal immune response while simultaneously supporting cell growth. Additional factors for consideration include compatibility of the biomaterial by-products, manufacturing ease and scaffold permeability to allow for exchange of nutrients and waste metabolites[47]. During the process of material selection, it is also critical to identify a biomaterial that can support the growth of the cell line chosen for the *in vitro* 3D model. Examples include the usage of fibrin and collagen to develop calcified tissue models or calcium phosphate and collagen for bone modelling[48,49]. The section below will highlight various polymers and their respective fabrication techniques.

3.1. Natural Biomaterials

The advantages of using 3D scaffolds composed of natural polymers include structural properties similar to the *in vivo* extracellular matrix (ECM), the presence of pre-existing ligands for cell surface receptors allowing for cell attachment and communication, as well as ease of fabrication. These properties are critical for cultured cells to proliferate and assemble a tissue in a biocompatible scaffold. Furthermore, natural polymers do not require post-chemical modification or synthesis to incorporate ligands or peptides, unlike synthetic polymers. However, there are a few disadvantages including weak mechanical properties, poor immunogenicity and uncontrollable degradation rates[50,51]. Examples of natural scaffolds include collagen, fibrin, gelatin, laminin, and Matrigel[52–55].

Matrigel is a commonly used natural ECM derived from the basement membrane of a mouse sarcoma. Therefore it resembles *in vivo* basement membrane and is composed of laminin, collagen IV and heparin sulfate proteoglycan as well as many growth factors, such as basic fibroblast growth factor (FGF), epidermal growth factor and insulin-like growth factor 1[56–59], with a limitation that the exact composition varies batch to batch. It has additional applications such as xenografting, invasion assays and angiogenesis tube assays[56,60]. Matrigel has been widely used for 3D culture for a variety of cells including breast cancer cells, human dermal papilla and hepatocellular carcinoma cells[46,61–63]. Despite successful application of this polymer, some disadvantages include high cost and the potential for xenogeneic by-products[64–67]. With these considerations, it is difficult to proceed with clinical applications since Matrigel is animal derived, incompatible with good manufacturing practices (GMP) and its batch-to-batch variability may influence disease modelling and drug testing results.

Another widely explored natural polymer is collagen gel derived from either rodent tails or bovine dermis, which recapitulates the *in vivo* ECM similar to Matrigel[68]. The advantages of this polymer include biocompatibility and tunable factors such as porosity and degradation rates[69]. Three examples of applications of collagen gels include the sandwiching of endothelial cells with other cells to fabricate capillary networks, smooth muscle-actin in fibroblasts or embryonic cardiomyocytes to build a new heart muscle model system[70–72].

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3.2. Synthetic Biomaterials

Examples of commonly used synthetic polymers include polydimethylsiloxane (PDMS), poly(lactic-co-glycolic acid), poly(ethylene glycol) and polyurethanes. Synthetic polymers are highly tunable to provide desired mechanical properties and degradation rates with improved reproducibility. They can also conjugate proteins or drugs for targeted delivery. The disadvantages include the lack of specific ligands for cell surface receptors, as well as potential toxic by-products produced by polymer backbone degradation[51]. To compensate for this, prior to crosslinking, the polymer backbone can undergo chemical modification with peptides such as RGD (arg-gly-asp) to enhance cell-specific adhesion or GPQG-AGQ to enhance proteolytic degradation or signalling ligands like vascular endothelial growth factor (VEGF) and bone morphogenetic protein (BMP) to regulate cellular activity[73–76].

PDMS is commonly used for the fabrication of microfluidic devices and organ-on-a-chip systems. Studies from a number of research groups have demonstrated the benefits to using this synthetic polymer in microfabricated devices due to its flexibility, low-cost, chemical inertness and relatively simple chemistry[77]. Despite advantages in microfabrication, there are a number of limitations of this material in 3D cell culture. One of the limitations is the affinity of PDMS for hydrophobic molecules which would hinder drug testing. One study proposed to coat PDMS with parylene or paraffin wax, however it is not the common practice in organ-on-a-chip devices[78]. Alternative materials with low absorption and adequate castability are still under investigation[79–82].

The focus of biomaterial research has shifted from natural to synthetic polymers that can match the biomimetic characteristics of natural polymers while allowing for greater reproducibility and control over the physical and chemical properties of the material. Hybridized synthetic and natural polymer scaffolds allow for researchers to draw on the strengths of both materials.

4. Modulating Growth Conditions to Support *In vitro* 3D Culture Systems

Decades of research on the embryonic development of several model systems has resulted in the ability to differentiate PSCs into lineages comprising all three germ layers. This developmental knowledge was employed to support the growth and expansion of patient-derived 3D culture systems such as spheroids and organoids. The precise control of cell signalling pathways known to regulate the fate of stem cells is a critical aspect in the development of *in vitro* 3D model systems. The development of novel technologies (Figure 2) such as bioreactors has allowed for the expansion of 3D tissues *in vitro* while facilitating the scaling up and long-term maintenance of cultures. In addition, novel organ-on-a-chip technologies (Figure 2) have allowed for multiple tissue systems to be connected *in vitro*, a step towards recapitulating *in vivo* physiological conditions.

4.1. Regulation of Developmental Signalling Pathways to Support *In Vitro* 3D Culture Systems

Several signalling pathways such as Notch, Wnt, Hedgehog and transforming growth factor β (TGF-β) are tightly regulated and play an important role during embryonic

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development[83–86]. Each of these pathways is known to regulate stem cell maintenance, differentiation and organogenesis in a context-dependent manner. During development, the activity of these pathways controls various key processes such as axis formation, germ-layer specification and left-right asymmetry. Nodal, a member of the TGF-β superfamily, is highly important as it regulates all three of these events[86]. TGF-β signalling, which includes the BMP and Activin subfamilies, is also critically important for organogenesis, since at least one ligand from the TGF-β superfamily is involved in the development of most organs[86]. The activity of these pathways is also significant for the homeostasis of many adult tissues, by regulating the proliferation and self-renewal of tissue stem cells as well as promoting injury-dependent organ regeneration.

It has become clear that these developmental pathways are connected, with activation of one pathway regulating the ligand expression of another. For example, Hedgehog signalling often induces the expression of Wnt ligands and in some cases BMP ligands[83]. Since these signalling pathways play important roles in stem cell maintenance and organogenesis, it is unsurprising that the use of stem cells as *in vitro* 3D model systems relies on the precise control of these pathways. Though some models rely on endogenous signals secreted by the cells in culture, often through co-culture of niche cells with stem cells, most *in vitro* 3D models utilize the addition of exogenous growth factors and small molecules. For example, the growth of small intestinal and colonic organoids requires the exogenous addition of Noggin, R-spondin and Wnt3A to inhibit TGF-β signalling and activate Wnt signalling[15]. Additionally, PSC-derived cerebral organoids resemble a dorsal forebrain fate if left untreated or can be patterned into a ventral forebrain fate upon the addition of SHH agonists and Wnt antagonists[87].

4.2. The Use of Suspension Cultures and Spheroids for *In Vitro* 3D Culture

The *in vitro* culture of non-adherent multi-cellular aggregates, or spheroids, is a commonly used example of a 3D culture model (Figure 2). Spheroids can be formed through several methods with the hanging drop, forced aggregation and centrifugation methods being the most known. The hanging drop method relies on the self-aggregation of cells within droplets, suspended from the surface of a well plate[88]. Commercial products such as AggreWell can be used to assist with initial spheroid formation within a standardized multi-well setting[89]. Alternatively, the liquid overlay technique forms spheroids by stirring the cell culture medium, usually within a bioreactor. Spheroid models have been generated for several tissue types including cartilage, pancreatic, muscle, retinal and brain[88]. Although spheroids are usually formed through aggregation of a single cell type, co-culturing different cell types can create more complex systems to better mimic *in vivo* tissues. Spheroid formation is reproducible and can be scaled up using high-throughput fabrication techniques[90]. One of the most common applications of spheroid cultures is the formation of neurospheres. Neurospheres can be generated from iPSC-derived neural progenitor cells, with the resulting spheroids resembling human fetal neural progenitor cells based on morphology and marker expression[6]. Spheroids can be used to model astrocyte maturation and development as demonstrated by the formation of human cortical spheroids from iPSCs, which were cultured for up to 590 days *in vitro*[91]. Liver bud spheroids were formed through co-culture of iPSC-derived hepatocyte progenitors with commercially available endothelial

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cells and mesenchymal stem cells[92]. These liver spheroids were shown to model *in vivo* fetal liver development based on transcriptome analysis. The co-culture of articular chondrocytes and mesenchymal stem cells isolated from osteoarthritic patients was used to develop an *in vitro* 3D model of cartilage[93].

4.3. The Use of Bioreactors to Support *In Vitro* 3D Culture Systems

Bioreactors are used for suspension cultures of various cell types and systems (Figure 2), including spheroids and organoids, allowing for improved nutrient and oxygen distribution that supports the expansion of 3D tissues *in vitro*[94]. Tutoplast, alginate and Matrigel are some of the most common polymers used due to their ability to mimic *in vivo* physiological properties including the presence of pre-existing ligands for cell adherence, communication via spatial and temporal signals, and structural complexity. Matrigel and alginate can crosslink to form hydrogels at various polymer concentrations, recapitulating the physical properties and structure of a human breast, used to study the cancer cell line MDA-MB-231[95].

Alternatively, synthetic polymers are advantageous as they are highly tunable to provide desired mechanical properties and degradation rates, are highly reproducible and can conjugate proteins or drugs for targeted delivery. Tillman *et al.*, have published their work on electrospun scaffolds used for vascular reconstruction composed of polycaprolactone and collagen. These scaffolds were advantageous due to their ability to support the growth of endothelial and muscle cells under constant pulsatile flow conditions and prevent *in vivo* inflammatory response for the duration of a month while maintaining biomechanical strength and integrity[96].

Bioreactors have been used for *in vitro* organoid systems such as retinal, brain and kidney in addition to suspension cultures of megakaryocytes and cardiomyocytes[10,97–100]. The use of 50mL rotating-wall vessel bioreactors to culture retinal organoids derived from mouse PSCs allowed for the development and maturation of retinal tissue that was not possible under static conditions[97]. Cerebral organoids were generated from iPSC-derived embryoid bodies embedded in Matrigel before transferring to spinning bioreactors[18]. The organoids were cultured for up to 10 months and expressed markers from all major areas of the brain as well as specific cortical sub-regions.

A 3D printed mini spinning bioreactor was developed to culture forebrain, midbrain or hypothalamic organoids *in vitro*[10,101]. This reusable device was smaller than most commercially available spin flasks, consisting of 12 chambers. However, the mini spinning bioreactor is not commercially available and requires in-house 3D printing to construct. A model of PSC-derived kidney organoids resembling the human fetal kidney was also developed using a 125mL spinner flask bioreactor[100]. The kidney organoids were comprised of nephrons containing podocytes, proximal and distal tubules, collecting ducts, endothelial cells and interstitial cells[100]. Spinner flask bioreactors can also be used to develop efficient, xeno-free and scalable culture systems, one example being the differentiation of iPSCs into megakaryocytes as a potential transfusion therapy for diseases such as thrombocytopenia[98]. Additionally, a 40mL bioreactor system was used to produce iPSC-derived cardiomyocytes in suspension culture[99].

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4.4. The Use of Microfluidics and Organ-on-a-chip Systems for *In Vitro* 3D Culture

An organ-on-a-chip is a type of microfabricated device used for culturing cells in micrometer-sized chambers with continuous perfusion of culture medium (Figure 2). The goal of organ-on-a-chip systems is to form a functional unit that replicates some aspects of *in vivo* tissues or organs. Multiple organ-on-a-chip systems can be linked fluidically to model the interface between tissues and the physiological interaction between different organs. Organ-on-a-chip systems can be used to quantify *in vivo* physiological functions of organs such as absorption of drugs and secretion of metabolites and enzymes[4,102,103]. Unlike static culture models, microfluidic systems incorporate physiological mechanical cues and fluid flow, which better support the growth of 3D tissues *in vitro* and help overcome the diffusion limit[104].

PDMS, as previously described, can be used for soft lithography to simulate vessels by forming narrow or wide channels while microcontact printing allows the cells to differentiate into the shape of choice. Using microcontact printing for drug screening, Khadpekar and colleagues demonstrated how C2C12 myoblast cells differentiate into circular or rectangular shapes and increase or decrease in size depending on the scaffold pattern[105].

A novel platform developed by the Radisic group called Biowire, incorporates PDMS channel templates which remodel type I collagen gels and seeded cells[106]. This method, in conjunction with electrical stimulation, assisted PSC-derived cardiomyocytes and supporting cells to develop electrophysiological properties and recapitulate cardiac tissues. In Biowire, cells were seeded into a microfabricated well containing a collagen gel surrounding a surgical suture. The cells were electrically stimulated with increasing frequency, which resulted in the generation of 3D aligned cardiac tissues with frequent striations. Due to the previously described limitations of PDMS with regard to drug testing, the next generation Biowire II platform, involved two parallel poly(octamethylene maleate (anhydride citrate)) (POMaC) polymer wires between which cardiac tissue would self-assemble[107]. This system allowed for continuous, non-invasive measurements of force and calcium transients. The addition of PSC-derived atrial and ventricular cardiomyocytes generated heteropolar Biowires that better mimicked *in vivo* tissue, allowing the system's application for disease modelling and drug. In addition, the platform supports the scale up production of therapeutically relevant cells for transplantation[108].

A microfluidic system was designed to model the blood-brain barrier *in vitro* through the culture of cerebral endothelial cells with neurons or astrocytes grown in separated compartments[109]. Furthermore, a microfluidic array system was used to co-culture human neural stem cells with human mesenchymal stem cells to investigate the paracrine signalling effects of mesenchymal stem cells on neuronal differentiation[110]. A model to study axon fascicles *in vitro* was created by culturing PSC-derived motor neurons in a microchannel device such that axonal growth was directionally restricted[111]. Morphological, electrical and physical analyses were performed using this system and hydrogen peroxide treatment was used as a proof-of-principle to model oxidative stress-mediated neurodegenerative disease.

An example of vasculature-on-a-chip is the AngioChip system, developed by the Radisic group, composed of a multidimensional scaffold containing parenchymal cells and a built-in

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endothelialized vascular network[112]. The AngioChip consists of a network of microchannels which are each 100 μm in diameter, distributed 10–20 μm apart, in order to overcome the diffusion limit of oxygen and nutrients within the parenchymal spaces. The use of AngioChip allowed for improved viability of parenchymal cells and vascular sprouting of endothelial cells from the lumen into the parenchymal space. An example of a multi-organ chip was created by connecting four human organ models using commercially available cell lines for the purpose of *in vitro* drug toxicity testing[113]. The multi-organ chip was composed of a 3D small intestine, skin biopsy, 3D liver spheroids and kidney proximal tubule compartments[113].

5. Developmental Models

In order to understand embryonic development, lineage specification and tissue homeostasis, developmental models of various organs are required. Furthermore, such models will provide insights into how certain diseases and drugs affect the developing organ.

In 2012, Sasai and colleagues asked whether an *in vitro* system of differentiated mouse ESCs could recapitulate some of the regulatory systems of organogenesis and form layered neural structures[114]. To guide self-assembly, this group explored the possibility of regulating spatial patterning and morphogenesis by employing developmental signals, developing methods to generate brain structures and the retina *in vitro*. More recent advancements to developmental models of the brain and retina will be described in the proceeding sections. Other developmental models, derived from endoderm and mesoderm fates, will also be discussed in brief.

5.1. Brain

Developmental models of the brain, in the form of 3D organoids and spheroids, have allowed researchers to investigate different elements of human brain development and pathology. Furthermore, such models can be used for testing novel stem cell and regenerative therapies of the central nervous system. The organization of the cerebral cortex is one of the characteristic features of cortical tissue architecture, including the formation of a polarized cortical plate and radially aligned neurons[115]. A goal of 3D culture modelling is to reproducibly generate human cortical tissue while maintaining this self-organizing capacity. Such models will allow for the study of early cortical development and neuronal migration. Signal modulation and scaffold design guide the differentiation of PSCs into neural tissue.

Different methods have been employed to recreate the detailed structural features of the brain, specifically during developmental stages. Silk-collagen scaffolds have allowed researchers to recapitulate the compartmentalized architecture and basic structural features formed during the development of the forebrain cerebral cortex, designing scaffold architectures that provided spatial separation of cell bodies and neural processes[116]. This structural foundation allowed for the development of robust neuronal projections and neural network maturation, when seeded with primary cortical neurons. Aside from structural stability, other properties of silk protein, such as its inherent negative charge that protects neurons from excitotoxicity, contribute to the longevity of the constructs. Alternatively,

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synthetic hydrogels have been a popular choice of scaffold in organoid development, as previously mentioned. Wu et al. investigated the role of hydrogel rigidity in the behaviour of human iPSC-derived neural progenitor cells (hiPSC-NPCs)[117]. Spontaneous migration and neurite out-growth were compared between soft and hard methacrylated hyaluronic acid (Me-HA) hydrogels. Soft hydrogels enhanced the neural differentiation of progenitor cells by upregulating the expression of neural maturation markers.

Despite the relative success of self-organizing *in vitro* cerebral organoids in investigating neural migration and network formation, limitations include high variability between batches, random tissue identity and incomplete morphological differentiation[118,119]. A recent study has suggested that inconsistent neural induction may be a main cause of organoid variability[115]. Neural induction refers to the process by which ESCs become committed to the ectodermal lineage and subsequently to a neural fate. Most recognized developmental models of the brain, published from 2008–2013, started from spherical embryoid bodies, which developed neuroectoderm on the exterior of the embryoid body[18,120,121]. To achieve consistent neural induction and improve the efficiency of neuroectoderm formation, a method was developed to increase the surface area to volume ratio of the engineered tissue[115]. Rather than spherical embryoid bodies, elongated embryoid bodies were generated from hPSCs seeded onto poly(lactide-co-glycolide) (PLGA) microfilaments as a floating scaffold. In contrast to spherical organoids, which displayed highly variable amounts of all germ layers, microfilament-engineered embryoid bodies were able to reproducibly form neuroectoderm with an almost complete lack of non-neural tissue[115].

Beyond the first report of neural rosette formation from human ESCs in 2001, current techniques are able to generate neural tissue which resemble the 3D organization of specific regions of the brain[7,122,123]. In 2008, Eiraku et. al established a method of generating self-organizing cortical spheroids using embryonic stem cells, mimicking early corticogenesis[120]. More recently, the possibility of generating neural tissue from human PSCs and fate-restricted neural stem cells have been explored. Although human iPSCs have been able to generate structures such as the cerebellum and cerebral cortex, the generation of more highly specialized structures seems to require a higher degree of pre-patterning. In an effort to address this challenge, human neuroepithelial stem cells have been cultured on Matrigel droplets and differentiated under dynamic conditions into human midbrain-specific organoids[124]. This method allowed for differentiation of neuroepithelial stem cell derived organoids into spatially organized midbrain dopaminergic neurons, forming clearly specified clusters within the human midbrain-specific organoids (Figure 2). Furthermore, a ventral midbrain identity was evidenced by the expression of midbrain specific transcriptional factors (e.g. OTX2) and nuclear factors (e.g. NURR1).

Advancements in 3D culture techniques have allowed researchers to model complex interactions and investigate the relationships between various subdomains of the developing human brain. Birey and colleagues modelled the saltatory migration of GABAergic neurons from the ventral to dorsal forebrain and their integration into cortical circuits, thereby monitoring interregional interactions with human cells[125]. As part of the study, dorsal and ventral forebrain subdomains derived from human iPSCs were fused *in vitro* to identify the

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transcriptional changes associated with interneuron migration and model diseases associated with migration defects, such as Timothy Syndrome. In another study, the concept of organoid fusion allowed for the generation of a dorsal-ventral axis by fusing independently patterned organoids of ventral and dorsal forebrain identities[87]. As was previously described, inconsistent neural induction often results in organoid variability. In order to promote a ventral forebrain identity, a combination of Wnt inhibition and activation of SHH signalling were applied to the human iPSCs during differentiation, to mimic *in vivo* embryonic development. Alternatively, a recent study examined interneuron migration by fusing human medial ganglionic eminence organoids and human cortical organoids[126]. To date, other models have lacked this complexity, rather focusing on generating brain organoids which model cortical development in isolation. The generation of a system to model human medial ganglionic eminence development is significant in that cortical interneurons are derived primarily from the medial ganglionic eminence of the subpallium.

The greatest challenge to be addressed in 3D developmental models of the brain is the generation of diverse cell types to mimic more closely the cell-cell interactions and circuit connectivity of the central nervous system. Furthermore, although methods have been developed to culture cerebral organoids for hundreds of days, the field requires robust methods for producing more mature tissue, beyond the fetal stages.

5.2. Retina

Many forms of blindness are caused by the loss or dysfunction of retinal photoreceptors. In order to better understand the development of such conditions, the key structural and functional features of the native retina are modelled *in vitro*. Recent developments in retinal organoids, primarily based on PSCs, contain not only physiological retinal cell subtypes but also model the *in vivo* layered morphology.

In vertebrates, the retina is developed from the neuroectoderm, specifically the anterior neural tube[127]. The optic vesicles are formed during the late stages of anterior neural tube formation. The distal region of the optic vesicles generate neural progenitor cells whereas the dorsal region gives rise to the retinal pigment epithelium[128]. The optic cup is formed by the invagination of the apical optic vesicles, such that it is brought into close proximity to the distal retinal pigment epithelium. Over time, the neural retinal progenitor cells give rise to all retinal neurons, including the cone and rod photoreceptors. The *in vitro* differentiation strategy is based on the idea of mimicking embryonic development by regulating similar signalling cues and growth factors as present *in vivo*. Differentiation requires neural induction of PSCs and neural patterning into the direction of the anterior neural plate.

Zhong and colleagues demonstrated, for the first time, the beginning of photoreceptor outer segment maturation, as well as some degree of photoreceptor functionality as evidenced by electrical response upon light exposure[129]. Human iPSC-derived free-floating aggregates are reported to successfully recapitulate the main steps of retinal development observed *in vivo* and form 3D retinal cups that contain all major retinal cell types with proper morphological arrangement. As shown in previous studies, Notch pathway inhibition is employed during the early to mid-stages of retinal development to increase the proportion of photoreceptors

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within laminated organoids, through a process of lamination in which neurons migrate towards their final layer within the developing tissue[130,131].

Despite taking initial steps to advance photoreceptor differentiation, the robustness of the photoreceptor outer segment was still lacking in the above study. The development of retinal organoids which give rise to photoreceptors with robust outer segments was first reported in 2017[132]. Through optimization of growth medium composition, oxygen concentration, and aggregate size, this study was able to form floating 3D optic vesicle organoids which supported long term culture and allowed for advanced photoreceptor development. Employing hypoxic conditions during culture reduced cellular stress, increased proliferation and enhanced chromosomal integrity, as previously demonstrated by the hypoxic generation of optic vesicles and optic cups as well as previous studies using 2D systems[133].

Researchers have also been working towards increasing the yield of PSC-derived retinal cells. In 2017, it was reported that 3D cultures with a biomaterial scaffold could improve the generation of retinal tissue from human PSCs. The use of hyaluronic acid or alginate-based hydrogels (0.5% RGD-alginate hydrogel) was able to enhance the yield of retinal organoids and the expression of neural retina and retinal pigment epithelium expression markers[134]. Although many studies to date have focused on the generation of retinal pigment epithelium and photoreceptors from PSCs, the derivation of retinal ganglionic cells has been largely limited due to the shortage of reliable markers for these cells. To overcome this limitation, Meyer's group undertook the comprehensive analysis of retinal ganglionic cell differentiation in order to enhance yield, by directing hPSCs to differentiate in a step-wise fashion towards a retinal lineage and identifying a specific subtype of retinal ganglionic cells, intrinsically photosensitive retinal ganglion cells[135]. This study was the first to demonstrate the development of intrinsically photosensitive retinal ganglion cells from human PSCs, as identified by the expression of the phototransduction protein melanopsin. Appropriate morphological and physiological features were also observed in the derived retinal ganglion cells at developmentally appropriate time points.

Despite the advancements that have been made in establishing physiologically relevant developmental models of the retina, there are a few challenges that still need to be addressed. Future research will work towards reducing culture time, developing photoreceptor outer segments with more mature morphologies, and the introduction of vasculature and immune cells

5.3. Endodermal and Mesodermal Models

Beyond cortical and retinal developmental models which are based on the ectoderm, PSCs are able to differentiate into endoderm and mesoderm germ layers, allowing for the development of organoid models of the intestine and kidney, respectively. Induction of PSCs towards endodermal or mesodermal lineages requires the Nodal signalling pathway; defective signalling compromises mesoendoderm development across all species studied[136]. Nodal activity determines the fate of the mesoendoderm, where high levels of signaling promote endoderm development and low levels promote the generation of the mesoderm. To mimic Nodal signalling, developmental models have utilized the TGF- β ligand, Activin A, to promote differentiation of PSCs[137].

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Activin A signaling results in expression of transcription factors Sox-17 and HNG-3- β, leading to posterior endoderm patterning, which gives rise to the small intestine. In 2011, Well's group constructed the first developmental model of the intestine by differentiating iPSCs into 3D human intestinal organoids[138]. Human intestinal organoids are capable of some transport functions and exhibit essential morphological features including the luminal brush border. However, a limitation of current models is that they fail to express proteins for specific segments of the small intestine (i.e. duodenum, jejunum, ileum and colon) due to their lack of maturity[139]. Rather, a mixed population of distal and proximal intestinal cells are produced. Efforts have been made to produce more mature intestinal models. In 2017, researchers developed a cross-linked collagen hydrogel which permitted stem cell maintenance and proliferation, as well as the formation of differentiated lineages of the small intestine[140].

Renal progenitors originate from the intermediate mesoderm and give rise to the various cell types of the kidney, such as pronephros, mesonephros and metanephros[5]. As per the developmental models described above, methods have been developed to generate differentiated progenitor cells from human PSCs. Due to the tissue complexity, researchers have been working towards identifying the developmental mechanisms responsible for specific cell types, each of which have distinct spatiotemporal origins from the intermediate mesoderm. Takasato and colleagues developed a method of selectively differentiating PSCs towards a collecting duct lineage, rather than kidney mesenchymal progenitor cells[141]. Using this methodology, anterior-posterior patterning of the intermediate mesoderm allowed for the formation of complex organoids with fully segmented nephrons, surrounded by endothelial and renal interstitium, effectively recapitulating first-trimester fetal kidneys[19,142].

Moreover, Takebe's group has contributed to the formation of vascularized 3D cultures and stable endodermal lineages. Although iPSCs are responsive to proliferative stimuli and ensure organ growth, chromosomal instability and variations in genetics or epigenetics makes them susceptible to tumorigenesis. Through reproducible generation of human posterior gut endoderm cells and utilization of FGF, TGF and Wnt signalling, progenitor cells formed multiple endodermal lineages with greater stability than iPSCs[143]. Large scale organoid production has been limited due to the high cost of developmental reagents required for nephron progenitor specification *in vitro*[141,144]. To alleviate this issue, a method has been recently developed to generate kidney organoids from iPSCs by substituting Fibroblast growth factor 9 with a more readily available reagent, Knockout Serum Replacement[100]. A challenge to be addressed in future studies is meeting mass transport limitations and organoid maturity.

The lateral mesoderm gives rise to several cell lineages, including cardiac progenitor cells. A crucial balance between Nodal and BMP2 signaling, regulated by morphogen gradients, allows for the mesendoderm to be segregated into a cardiac mesoderm[145]. However, the signals that trigger the migration of cardiogenic mesodermal cells and determine early cell fate decisions – from epiblast to specific cardiac cell – remain unclear. Recently, genome editing has allowed for the study of human cardiogenesis: the identification of networks of genes that drive specific differentiation events[146].

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6. 3D Cultures for Disease Modelling

The advent of commercially available stem cell lines and molecular biology techniques such as single-cell RNA sequencing, cryo-electron microscopy and the CRISPR/Cas9 technology have resulted in a paradigm shift in bioengineering. Researchers are now capable of recreating patient-specific pathologies *in vitro* to understand disease etiology and progression, while additionally testing drug candidates for treatment. The limitations associated with animal models and immortalized cell lines such as cost, ethical concerns and poor recapitulation in clinical trials will likely no longer be obstacles in the future of medical research. These 3D culture models will allow clinicians to come closer to determining the molecular basis of a disease rather than relying on established signs and symptoms for diagnostic purposes.

The current gold standard for preclinical testing and disease modelling is animal studies. However, such testing is liable of inadequate clinical translation since mechanistic findings differ considerably across species. Additionally, the conditions of animal testing often cause distress as well as uncontrollable and abnormal changes in behaviour, raising a number of ethical concerns[147]. To address this need, complex control and monitoring systems have been engineered to recapitulate a physiological microenvironment providing stimuli to induce targeted effects in *ex vivo* models as summarized in Table 3[148]. Genomic tools have elucidated key pathways that can trigger the onset of disease in healthy tissues, narrowing down the search for therapeutic targets and effectively reversing a disease state.

6.1. Central Nervous System and Eyes

Most of the recent studies in 3D culture have focused on the central nervous system. The complexity of the human neural network and the tight regulation under which it functions have left researchers with many unanswered questions. In the past, ethical limitations have restricted scientists, solely permitting the analysis of patients post-mortem and animal models with inherently simpler cerebral systems. The need for a more accurate human model has driven many to create both *in vitro* and *in silico* models for testing[149].

By reprogramming stromal tissues gathered from a patient with severe microcephaly, researchers have conclusively demonstrated that mutations in the *CDK5RAP2* protein cause premature neural differentiation and a loss of key progenitor cells[18]. Additionally, deviations observed in the orientation of radial glial spindles resulted in a lack of symmetry which stunted neural stem cell growth.

The high incidence of neuropsychiatric diseases such as autism spectrum disorder, bipolar disorder and schizophrenia present a serious burden to modern medicine. Current findings indicate that there is a substantial overlap in causative mechanisms for these disorders, but discrepancies in different risk-associated genes and sensitizing environmental effects result in a variety of disease phenotypes. By harnessing the computational power of large-scale molecular profiling techniques, such as single-nucleotide polymorphism typing and exosome sequencing, a comparison of cell types and their hierarchical organization can be assessed for key disorder-specific determinants.

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However, the maturation of neural circuits and heterogeneous cell networks has yet to be validated against adult tissues and will require further investigation[149]. In 2014, Choi *et al.* developed the first human neural model which demonstrated both critical hallmarks of Alzheimer's disease: β-amyloid (Aβ) plaques and neurofibrillary tangles. This was utilized to understand the molecular mechanisms of p-tau pathologies that would generate Aβ species even in the absence of mutations in the frontotemporal lobe. FAD mutations in the β-amyloid precursor protein and Presenilin-1 gene were tied to the attenuation of plaque formation and could prove to be a viable target for therapeutic intervention[150].

It has been nearly a decade since the first 3D self-organizing optic cup was developed by Eiraku and colleagues using floating aggregates of ESCs cultured in serum free, low growth-factor conditions[151]. This resulted in the formation of a two-walled cup morphology with an invaginated distal section that expressed neural retinal markers (Chx10, Rx, Pax6 and Six3), within a culture period of only 8–10 days. Breakthroughs such as this have spurred scientists to explore other aspects of ophthalmic architecture such as the human trabecular meshwork (HTM), which is a primary risk factor in glaucoma due to its ability to regulate intraocular pressure. Cultures of patient-derived HTM cells were subjected to 7 days of prednisolone acetate treatment, a corticosteroid that is administered to treat swelling within the eye, followed by perfusion and immunohistological studies[152]. An increased expression of myocilin and ECM proteins was observed, resulting in the formation of fibrous plaques. This coupled with higher resistance due to structural changes in the trabecular meshwork and the accumulation of ECM by-products linked to the downregulation of matrix metalloproteinases, suggests a possible mode for disease progression. 3D culturing techniques have allowed for the development of structured retinal sheets for transplantation to help treat patients suffering from retinitis pigmentosa (RP), which leads to blindness due to the loss of photoreceptor cells. Although this disease is genetic in nature, the complex pathology and the large array of mutations associated with RP have ruled out the use of gene therapies as a primary treatment option. However, recent work conducted in the RIKEN Center for Developmental Biology demonstrated the successful subretinal transplantation of iPSC-derived 3D retinal tissues. These constructs, containing mature photoreceptors integrated with the host bipolar cells, could have a potential application in studying disease progression *ex vivo* and investigating the genes responsible for retinal degeneration[153].

6.2. The Heart and Lungs

As discussed throughout the review, 3D cell culture systems allow for cells to be exposed to specific biomechanical stimuli necessary for expression of physiologically relevant phenotypes, given their tight control mechanisms and capacity for longitudinal study. One dynamic study combined genetic manipulation as well as chemical and mechanical stresses to investigate the pathology of dilated cardiomyopathy. Findings indicated that titin-truncating variants and related missense mutations can diminish contractility in cells, while associated protein mutations interfere with regeneration and remodelling of cardiac tissues. This can lead to reduced responses to myocardial stresses, which are critical for maintaining cardiac health[154].

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The functional maturation of engineered tissues is a fundamental challenge of tissue engineering. A study conducted by Tiburcy *et al.* was capable of not only displaying key characteristics of mature myocardium but also producing a response similar to cardiac failure (cardiomyocyte hypertrophy, contractile dysfunction and N-terminal pro B-type natriuretic peptide release) when experiencing long term catecholamine toxicity[155]. Given that the cells utilized in this study were patient-derived, it is highly probable that patient specific myocardium models could be generated for personalized drug testing.

Biowire II, as described above, has demonstrated the capacity for the controlled assessment of mechanical, electrophysical and polygenic disease modelling. A study comparing the profiles of RNA expression from Biowire II models of both diseased and healthy patients displayed a consistent upregulation of 25 pathways linked to the pathological remodelling of cardiac tissues. Significant differences were also observed in the contractile function of the engineered tissues[3,156]. Likewise, studies have demonstrated that PSC-derived human cardiomyocytes can display *SCN5A* and Na^+ channel repression mutations which may be used to model cardiac Na^+ channel disease[157]. These are just some examples of the many research findings that could be harnessed to develop functional disease models for human cardiology.

In the last decade, organ-on-a-chip technologies have heavily contributed to our understanding of cellular interactions and behaviours under defined environmental conditions. A model of the alveolar-capillary interface containing human pulmonary epithelial and endothelial cells was able to recreate breathing motions through changes in air and fluid flow as well as induced mechanical strain. This was later used to test for pulmonary edema induced by drug toxicity through the administration of clinically relevant doses of interleukin-2 (IL-2), an FDA approved oncological drug. The combined effect of IL-2 and simulated cyclic mechanical strain from breathing lead to measurable disruption of the alveolar epithelium and capillary endothelium, substantiating the toxic effects of such a treatment.

Protocols published in 2014 by researchers from the Columbia Center for Translational Immunology provided detailed accounts of the agents necessary to convert human iPSCs into basal, goblet, club, ciliated as well as type I and II alveolar epithelial cells. With access to this large catalogue of cells, a number of unique studies could be proposed to assess different co-cultures and cellular interactions in relation to critical pulmonary diseases[158]. Another study which provided a better understanding of the synergistic relationship between the epithelial and mesenchymal linings of the lungs, involved the co-culture of bronchial epithelial cells and lung fibroblasts on a collagen gel matrix. This was used to study tissue and ECM remodelling upon the delivery of transforming growth factor β1 (TGF-β1) which is known to promote remodelling of connective tissues and plays a critical role in maintaining the epithelial defenses against viral infections, allergens and pollutants[159]. Astonishingly, there is no robust *in vitro* model for measuring the infectivity of viral agents in humans. Such models would greatly help to accelerate therapeutic discoveries as they could unearth the mechanism of infection and replication of the virus in humans. Work carried out by Zhou and colleagues at the University of Hong Kong allowed for the development of long-term expanding 3D lung organoids, which replicated a human airway

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inclusive of four cell types: goblet, ciliated, club and basal cells[160]. A cell culture medium, consisting of CHIR99021, a Wnt agonist, and FGF10 was employed to increase the number of ciliated cells and the level of serine proteases in the system, necessary to induce viral infection. Thus, such a model could be applied in future studies to investigate differences between strains of the influenza virus and other pulmonary infectious agents.

6.3. Abdominal Organs

Similar approaches, as discussed above, have been applied to study larger organs in the abdominal cavity. Human iPSCs were programmed into hepatocyte-like cells and cholangiocytes to develop liver organoids[161]. The advantage of this technique is that the cells were sourced from skin fibroblasts and mononuclear blood cells, which can be collected in a relatively non-invasive manner. The use of gene editing would make these models robust tools for the study of monogenic liver diseases, while integration with other organ models may help to determine how dysfunction in the liver can directly affect other organs.

To this purpose, work carried out at Imperial College London lead to the development of a 3D microfluidic primary human hepatocyte culture, capable of maturing over 40 days, which was a period long enough to model the life cycle of Hepatitis B virus (HBV) infection and replication[162]. This helped elucidate the mechanisms by which HBV suppresses the innate immune system and identified novel biomarkers that may correlate to the diseased state. This novel culture system can be combined with viral agents isolated from patients for the *ex vivo* investigation of potential therapeutics.

Similarly, Freedman *et al*, demonstrated that human iPSCs are capable of recapitulating renal tissues through the development of self-organized hollow spheroids[163]. The inhibition of the enzyme glycogen synthase kinase-3 resulted in segmentation within the spheroids to create cells characteristic of podocytes, endothelium and proximal tubules. The organoids expressed appropriate biomarkers such as kidney injury molecule-1 (KIM-1) upon the induction of nephrotoxic chemical injury due to the administration of cisplatin or gentamicin. Additionally, a number of genetic studies have recently surfaced due to the increased accessibility of gene sequencing tools.

Forbes *et al.* examined a kidney organoid platform developed from a patient identified with nephronophthisis, a disease that impairs renal function in children causing premature kidney failure[164]. This study identified a mutation in IFT140, a gene tied to retrograde intraflagellar transport and determined that diseased individuals expressed significantly lower levels of genes for cell-cell junction, dynein motor assembly and apicobasal polarity. Recently, an *in vitro* co-culture model of the interface between tubular epithelial cells and neighbouring fibroblasts was used to examine, in real time, the compounds that could modulate intercellular crosstalk in patients suffering from kidney fibrosis[165]. Though limitations in replicating the synergistic and complex effects of the native environment remain, the novelty of this approach lies in the high-throughput manner by which it can record related findings that may conceivably be extended to pulmonary fibrosis.

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Pancreatic organoids developed from human iPSCs have generated tissues containing acinar and ductal cells which exhibit appropriate marker profiles, gene expression and functional hallmarks. Upon implantation into mice, these tissues have shown no evidence of tumourigenic capability and have further been used to study the effects of cystic fibrosis on pancreatic function[166]. This research was able to correlate cystic fibrosis transmembrane conductance regulator (CFTR) activation levels in functional assays to global expression in the body and develop an mRNA-based gene therapy for the treatment of diseased tissues.

6.4. Multi-Organ Systems

Although much progress has occurred in disease modelling, one major challenge is developing models of systemic diseases such as Lyme disease, atherosclerosis, malaria and diabetes[167]. In systemic diseases, no effect is isolated, but rather has a cascading impact in related organ systems. To address this, some researchers have developed culture systems with multiple organs modelled in sequence[168]. Although the scale of the network developed may be limited, novel interactions between tissues can be elucidated.

Maschmeyer *et al.*, presented a functional four organ system consisting of human intestine, skin, liver and kidney counterparts, held in co-culture for 28 days[113]. The biological barriers of each organ were tested for sustained integrity over the duration of the study and the model was used to test drug uptake and delivery. Such a system could also be applicable for studying the chronic effects of systemic diseases or the spread of viral agents throughout the body. Likewise, researchers at the University of Central Florida developed a system containing cardiac, muscle, neuronal and liver modules with pumpless recirculation of media and continual functionality over 14 days[169]. This arrangement allowed for the testing of mechanical, chemical and electrical responses to therapeutic agents in order to assess their toxicity. Although such models are currently utilized for drug testing, they may have a significant potential in the assessment of disease progression in patients. Such models will provide a better understanding of complex diseases and allow for a multifaceted therapeutic approach.

Even in these advanced bioengineered systems, challenges related to the accurate recapitulation of physiological conditions still exist. One such task is the design of a blood substitute that can circulate through the system carrying nutrients, biomolecules and waste to support crosstalk between tissues. This material would likely have to be renewable with a carrying capacity similar to human blood. The use of synthetic materials in these models may also be a source of concern as their long-term effects on the cells in culture and the possible drugs or pathogens introduced would need to be assessed for clinical relevance.

7. Drug Testing

The importance of 3D cell culture models in drug testing has increased over the past decade due to the ability to recapitulate some characteristics of *in vivo* conditions. 3D cultures can act as a preclinical model to determine cellular responses to drugs or for personalized medicine with patient derived cells. The use of 3D cultures for *in vitro* drug testing has a potential to better translate to patient findings than the previously used animal models, improving the success rate of future clinical trials. For example, Theralizumab (also known

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as TGN1412), a novel monoclonal antibody drug, was used to stimulate and expand T cells independently of the receptors. The first phase I clinical trials took place with six healthy patients where they were intravenously injected to assess the effects. Within 90 minutes, all six patients had systemic inflammations that led to the failure of several organs. Luckily, after intensive organ support, all six patients survived[170]. If this drug could have been tested for side effects using an *in vitro* 3D model, the severe complications that occurred to those six patients could have been prevented. This section will focus on the validation of 3D culture models and monitoring drug responses for disease models.

There is a particular interest from the standpoint for drug discovery in developing a blood brain (BBB) barrier models, as permeability of this barrier critically determines central nervous system toxicity of new drug candidates. In addition, with the prevalence of cancer, there is an urgent need to develop safer and more effective cancer drugs. Thus, current research efforts have focused on developing new BBB and cancer models. In addition, cardiac toxicity remains the predominant reason for the withdrawal of already approved drugs. This paper will focus on reviewing the development of these models from human cells in the context of drug discovery.

7.1. Blood Brain Barrier

3D cell culture models must be validated by observing the interaction between drugs of known effects and cells, in order to determine if these models recapitulate human physiology. There have been several well-established 3D models recapitulating many parts of the body including the human lung, pancreas, liver, intestine and bone[77,171–174]. A part of the body that has yet to be completely replicated and understood is the blood-brain barrier (BBB), the most complex neuroprotective barrier between the blood and the brain. The BBB allows only specific molecules to pass through the membrane, which prevents the entry of many therapeutic candidates developed to treat central nervous system (CNS) disorders. Many studies have attempted to recapitulate the BBB, yet limitations remain. One of these limitations includes the loss of human brain microvascular endothelial cell characteristics in culture, due to the use of pump systems to deliver nutrients in microfluidic devices. Additional constraints include models with high fluid-to-tissue ratio and the utilization of animal brain tissues (i.e. murine), which do not accurately represent the human BBB.

Wang et al., developed a pumpless microfluidic BBB model which incorporated a permeable layer, with minimal applied shear stress[175]. This model co-cultures rat primary astrocytes and hiPSC-derived brain microvascular endothelial cells on silicone sheets, sandwiched between porous polycarbonate membranes. The study used FITC-dextran, doxorubicin, cimetidine and caffeine to validate their BBB model to determine if they resembled *in vivo* conditions[175].

Campisi et al., was the first to design a BBB microfluidic model from PDMS using a tri-culture of primary brain cells, human primary astrocytes and microvascular cells derived from human iPSC-derived endothelial cells. These cells contribute to the overall integrity of the BBB and facilitate the passage of molecules across the barrier. The incorporation of these cells was validated by staining for specific junctions (ZO-1, occluding and calduin-5), laminin and collagen IV captured by confocal images. To further validate the model, the

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permeability coefficient for 40 kDa and 10 kDa FITC-dextran was compared to *in vivo* rat conditions[176].

Lastly, *Wevers et al.*, developed a high-throughput microfluidic platform that cultures endothelial cells, pericytes and astrocytes on a two lane OrganoPlate along with a collagen-I gel[177]. Similar to the papers previously mentioned, the model was validated via immunostaining of tight junction proteins and incorporating FITC-dextran to determine the permeability coefficient. The authors proceeded to test for MEM-189, an antibody that binds the human transferrin receptor which is expressed by endothelial cells, with anti-hen egg lysozyme serving as the negative control due to its inability to bind to human cells[178,179]. Results obtained from flow cytometry demonstrated anti-human transferrin receptor bound to the endothelial cells as expected from *in vivo* studies. This model has potential for the discovery of CNS-penetrant antibodies[177]. As these novel techniques are published, this will bring us closer to recapitulating the complex structure of the BBB in order to understand which drugs can be effectively used to treat diseases like Parkinson's disease, Alzheimer's or brain cancer.

Once a 3D cell culture model has been validated as mentioned above, drug screening can be completed to determine the cellular response. The goal of drug screening is to test multiple drugs to find a candidate that can decrease the viability or proliferation of diseased cells. These candidates can then be further tested with additional 3D *in vitro* or *in vivo* animal models to confirm the effects of the drug prior to clinical trials.

7.2. Brain and Eye Disease

Recent work has contributed to the development of 3D models capable of replicating Alzheimer's disease. Clinical trials for docosahexaenoic acid (DHA) as a potential treatment for Alzheimer's disease (AD) have shown variable results[180,181]. A study by *Kondo et al.* utilized iPSCs from AD patients with either an autosomal-recessive mutation of the APP-E693 gene or sporadic AD cells. The iPSCs were differentiated into neural cells using a 3D floating culture, which was further differentiated in Matrigel. The results showed that higher concentrations of DHA decreased the levels of proteins prevalent in AD like BiP, cleaved caspase-4 and peroxiredoxin-4. In addition, stress oxidation with the CellROX™ assay, decreased the progression of AD, as previously demonstrated[182–184]. Finally, the LDH assay showed >80% viability for up to 16 days when exposed to the drug. Despite the promising results, DHA treatment did not decrease the levels of Aβ. Therefore, the authors concluded that evaluating more patient-derived AD cells at various stages of disease was required to understand why the Aβ levels remained constant after DHA treatment[185]. This model will help clinicians understand the complex mechanisms of AD development and potentially find a candidate to treat this disease.

In terms of developing 3D models of eye disease, *Torrejon et al.*, focused on understanding the effects of rho-associated kinase (ROCK) inhibition on patients with glaucoma, as this drug is currently in clinical trials to determine if the drug action disrupts the actin filaments in the human trabecular meshwork (HTM) of the eye. By adding ROCK inhibitors, the levels of collagen IV, myocilin and fibronectin substantially decreased supporting the use of this drug in clinical trials. Although this paper did not discover a novel drug, the ability to

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recapitulate the glaucoma with steroid treatment is novel and can be used as a platform to test other drug candidates[186].

The eye and brain models selected for this review focused solely on glaucoma and Alzheimer's disease. What is missing is the ability to simulate multiple diseases under one model. Despite the success in developing these novel models, one current limitation is to determine and evaluate which specific model most faithfully recapitulates the cellular response upon drug treatment. Once these organ models are fully validated, the next step for drug testing is to connect multiple organs together, an advance which will require a thorough understanding of scaling laws between different organs. Recent reviews highlight the challenges and potential solutions to achieving this task[187,188].

7.3. Cancer Models

Cancer has been an ongoing issue that patients encounter with few to no solutions, leading to an abundance of cancer models that have been published within the past 5 years. These models tested drugs which showed promising results in clinical trials and animal models, providing another platform to re-evaluate previous findings and elucidate the mechanisms of action. In the proceeding sections, we will highlight various oncology, neurologic and cardiac cancer models.

A brain cancer chip was established by developing a 3D microfluidic device comprised of a photopolymerizable hydrogel composed of poly(ethylene) glycol diacrylate (PEGDA) because of its ability to release drugs through diffusion[189]. A commercially available glioblastoma cell line, U87, was grown in a 3D spheroid and cultured in the PEGDA hydrogels to form cancer spheroids which were tested with Pitavastatin and Irinotecan. These FDA-approved drugs are used to treat glioblastoma multiforme (GBM). This unique microfluidic system can administer these drugs together through separate channels where each channel receives a different concentration of the drug in a system. The results indicated equal concentration of Pitavastatin and Irinotecan injected together had the lowest cell viability after 1, 4 and 7 days administered in comparison to the drugs individually.

A 3D organoid culture system was generated using donor-derived liver tissues to simulate near-physiological conditions that retained liver functionality[190]. This model was used to study primary liver cancer (PLC) and tested 29 anti-cancer compounds in a dilution series of each drug, where a dose response curve was completed to determine the viability utilizing luminescent single curve[191]. Of the 29 compounds, the drugs with promising results were Taselisib, Gemcitabine, SCH772984 and Dasatanib based on reduced cancer cell viability. Specifically, Taselisib inhibited the growth of the tumoroids, while Dasatinib suppressed tumoroid formation. The remaining drugs tested did not affect PLC viability[190]. Thus, the next step is to use 3D culture models to evaluate combination therapies to look for synergistic effects.

A 3D pancreatic cancer model grown in Matrigel was used to test six chemotherapeutic drugs: gemcitabine, paclitaxel, docetaxel, doxorubicin, epirubicin and vinorelbine[192]. Gemcitabine is the current standard treatment for pancreatic cancer, however it has limited efficacy[193]. To determine the efficacy of each drug, a resazurin assay and cell viability

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staining (calcein-AM) were applied to the cells to determine the survival of cells treated after 6 days at a drug concentration of 200μM and 0.2 nM. Treatment with gemcitabine decreased cell viability as shown in the resazurin and cell viability assays as predicted, while none of the remaining drugs tested showed similar results. This indicates the importance of finding novel drugs to treat pancreatic cancer, with additional options including combining multiple therapeutic agents to further decrease the viability of cancer cells[192].

A developed microfluidic lung-on-a-chip model was simulated by culturing non-small-cell lung carcinoma (NSCLC) adenocarcinoma cells with primary lung alveolar cells or small airway epithelial cells on the top channel, while the bottom channel contained endothelial cells. Adjacent to the endothelialepithelial complex were vacuum channels to replicate the cyclic mechanical strain or breathing patterns. Next, two known drugs, rociletinib and erlotinib used in clinical trials for NSCLC were exposed to this model to determine cell viability via a MTT assay[194–196]. In this specific paper, the rociletinib-treated cells showed almost no survival at a concentration of 1μM while erlotinib treatment decreased survival by 40% at 10 μM. These drugs were then introduced to the microfluidic device to replicate these results, but there was no decrease in tumour viability. These results contradicted the expected outcomes from previous work and more importantly from clinical trials, thus, emphasising the importance of critical evaluation of results obtained from 3D cell culture systems[197].

7.4. Cardiac models

Cardiac models have been heavily studied for drug modelling as there are numerous diseases affecting the heart, as previously mentioned. An example of a 3D drug screening model is the three dimensional cardiac model fabricated by Lu et al., that tested antibiotics (ampicillin, erythromycin and Trovafloxacin), anticancer (Vandetanib and Tamoxifen) and drugs for treating diabetes (Metformin, Rosiglitazone and Troglitazone)[198]. The ATP activity of the cells and tissue contractibility determined the overall effect of the drug and was compared to clinical results. This model demonstrates how the tissue's contractility could detect cardiotoxicity more effectively than the ATP activity of the cells. In fact, six of the eight drugs showed results similar to clinical observations, a sign of promising preliminary results for the model.

The Biowire platforms, described in the previous section, can be used for drug screening[3,106]. This was shown by exposing the model to known drugs with chamber-specific responses, including Ranolazine for decreasing conduction velocity and serotonin for increasing the Ca^{2+} transients in the atrial regions while not affecting ventricular regions. More importantly, Biowire II can be used to understand if the drugs tested can have a synergistic or independent effect on the atrial and ventricular regions[3]. It has also been used for screening of small molecule kinase inhibitors for cardiotoxicity.[199]

8. Summary

2D cell culture models generally lack the architecture of *in vivo* tissues. Intense research over the past decade has resulted in the development of many 3D culture models that recapitulate aspects of *in vivo* organ structure and function. In particular, the development of

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organoids has revolutionized developmental biology, disease modelling and drug discovery as organoid models are capable of generating complex 3D structures that contain multiple cell types and retain some functions of the *in vivo* organ.

The introduction of novel biomaterials has been critical to the development of *in vitro* 3D models. Further studies in the field of biomaterials should focus on the generation of hybrid polymers, combining the advantages of both natural and synthetic polymers, to replace the use of common materials such as PDMS and Matrigel. The use of PSCs and ASCs has allowed for the generation of models that span all three germ layers and most organs. However, the current challenge with most directed differentiation protocols is the fact that they result in cells and tissues that resemble embryonic and fetal tissue rather than the mature cells required for transplantation. Furthermore, the utilization of bioreactors allows for scaling of production, which is needed for the translation of these 3D systems into cell therapies. Additional research is required to connect multiple organ models for *in vitro* disease modelling and drug testing in a body-on-a-chip system. Another important challenge is to improve the reproducibility of current models, especially organoids, which can be achieved through applying engineering methods of microfabrication to control the cellular microenvironment.

The generation of 3D culture systems has greatly enabled the study of human development *in vitro* while minimizing the ethical considerations, allowing for the investigation of early embryonic development, lineage specification and organogenesis. *3D culture models* have been critical in studying aspects of human development which have been previously limited by interspecies differences, providing a better understanding of congenital conditions, developmental disorders, and human diseases. Current disease models have mainly focused on examining the effects of a disease on an individual organ. Additional research is required to integrate the various organ models through a body-on-a-chip approach to study the systemic effects of disease. The generation of 3D culture systems has also revolutionized *in vitro* drug testing. 3D systems will replace traditional 2D culture systems for high-throughput screening of compound libraries and allow for greater clinical translation due to the improved physiological relevance of these models. The development of 3D models that allow for *in vitro* testing of drug absorption, metabolism and toxicity will minimize the need for expensive pre-clinical animal studies. Importantly, the use of iPSC-derived 3D systems enables personalized medicine by performing drug screens with patient-specific cells to inform the treatment plan for that patient.

The generation of *in vitro* 3D models that mimic *in vivo* organs has great potential for the discovery of novel therapeutics, including small molecules and cell therapies, as well as understanding developmental mechanisms of diseased and healthy tissue. There is also a potential to commercialize these models, in particular for the various organ-on-a-chip and body-on-a-chip systems that can be mass produced for both academic and industrial purposes. By developing more representative culture systems, clinicians and scientists will be better able to diagnose, treat and mitigate disease.

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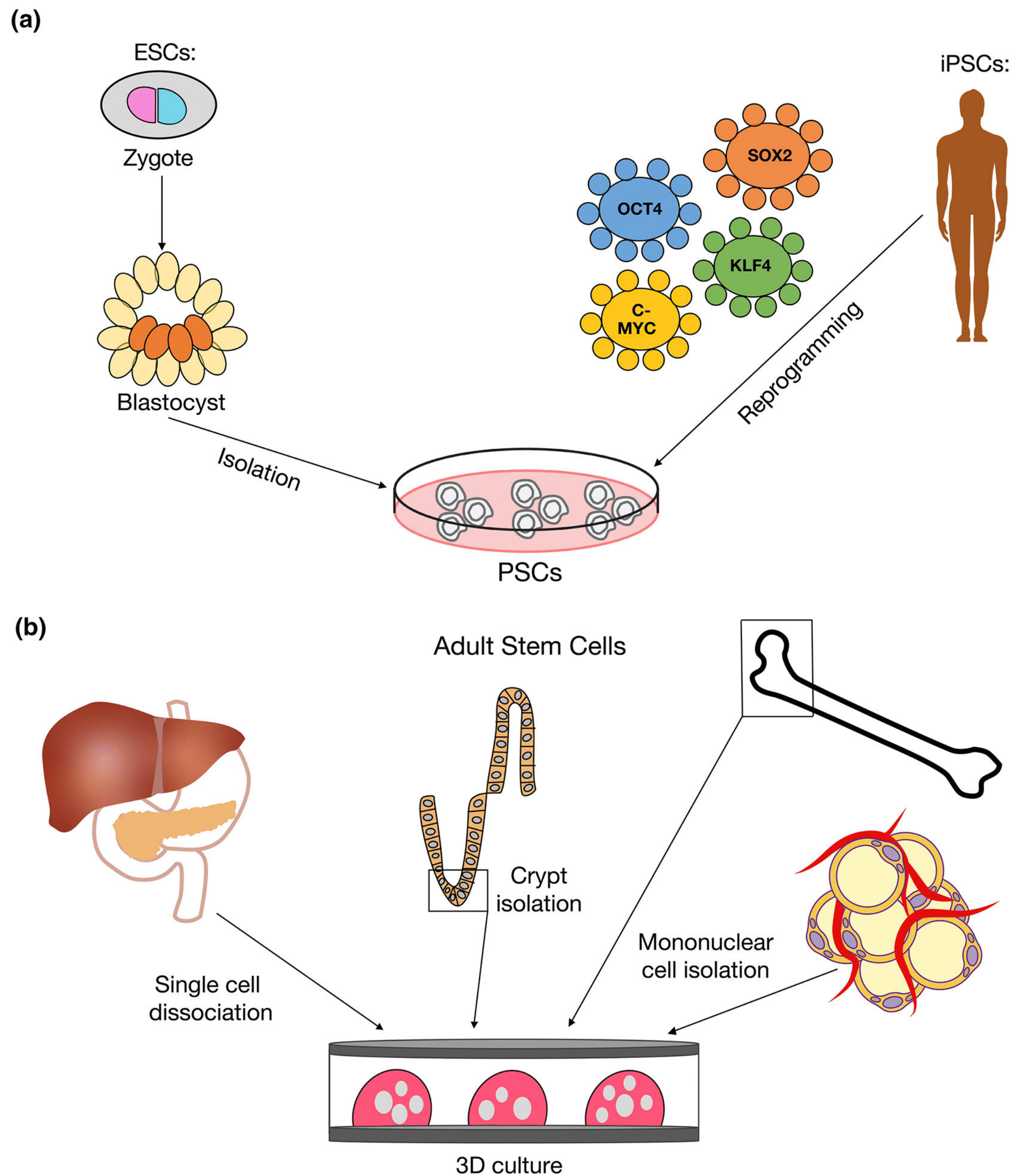

Figure 1. Sources of cells for in vitro 3D culture models

A. Human PSCs are comprised of ESCs and iPSCs. ESCs are generated from pre-implantation embryos by isolating the inner cell mass of a blastocyst and culturing these cells *in vitro* under the appropriate conditions to maintain pluripotency. Alternatively, iPSCs are generated by the direct reprogramming of adult cells into a pluripotent state. A viral vector encoding the transcription factors OCT4, SOX2, KLF4 and c-MYC, known as the Yamanaka factors, is used to deliver the reprogramming factors to the cells. **B.** Adult stem cells (ASCs) isolated from healthy volunteers or patients are grown *in vitro* under optimized conditions to support the growth and expansion of 3D culture models such as organoids and spheroids. Examples of tissues where ASCs have been successfully isolated from include liver, pancreas, intestinal crypt, bone marrow and fat[15,31,37,39,40,93].

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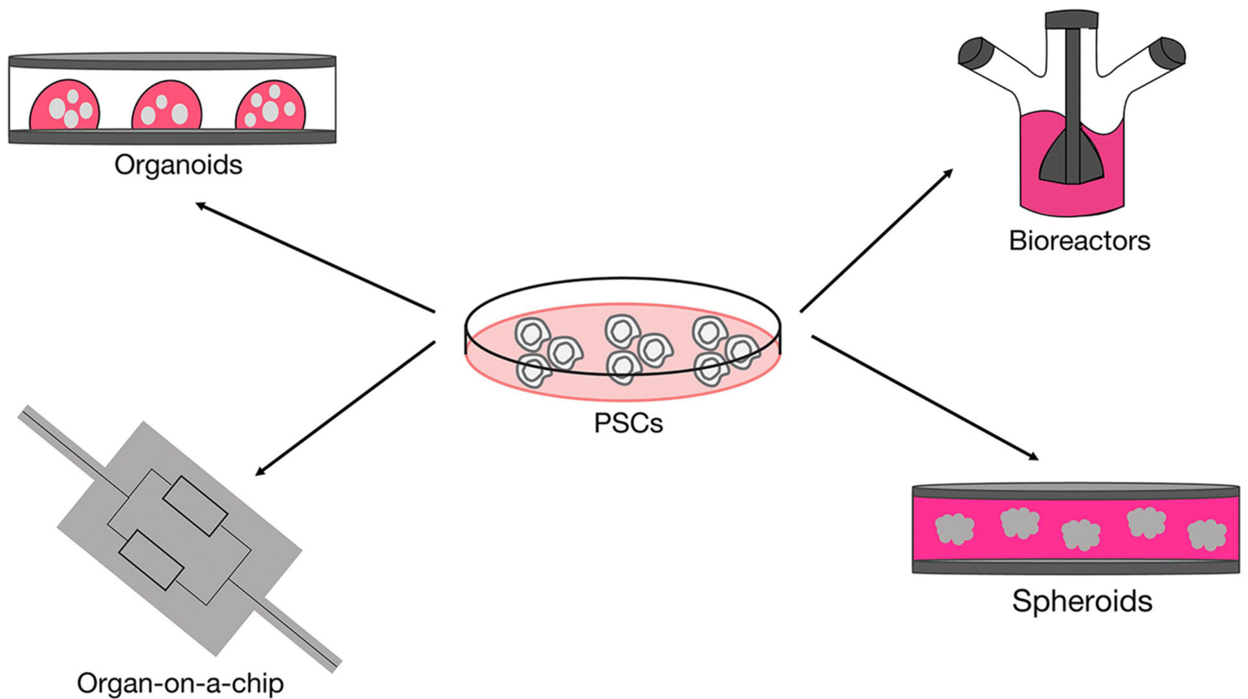

Figure 2. Technologies to support in vitro 3D culture models

PSCs can be used to generate a range of different 3D culture models by combining differentiation protocols with technologies to support cell growth under 3D conditions. The simplest method involves culturing differentiated cells under suspension conditions to form spheroids. Organoids are generated by embedding differentiated cells in an extracellular matrix such as Matrigel. Bioreactors are used to scale up organoid and spheroid cultures and to allow for long-term growth of these tissues due to increased circulation of nutrients and waste products. Organ-on-a-chip systems make use of microfabricated devices with micrometer-sized chambers and continuous perfusion of culture medium. Multiple chips can be linked fluidically to join several organ models and recapitulate *in vivo* physiology.

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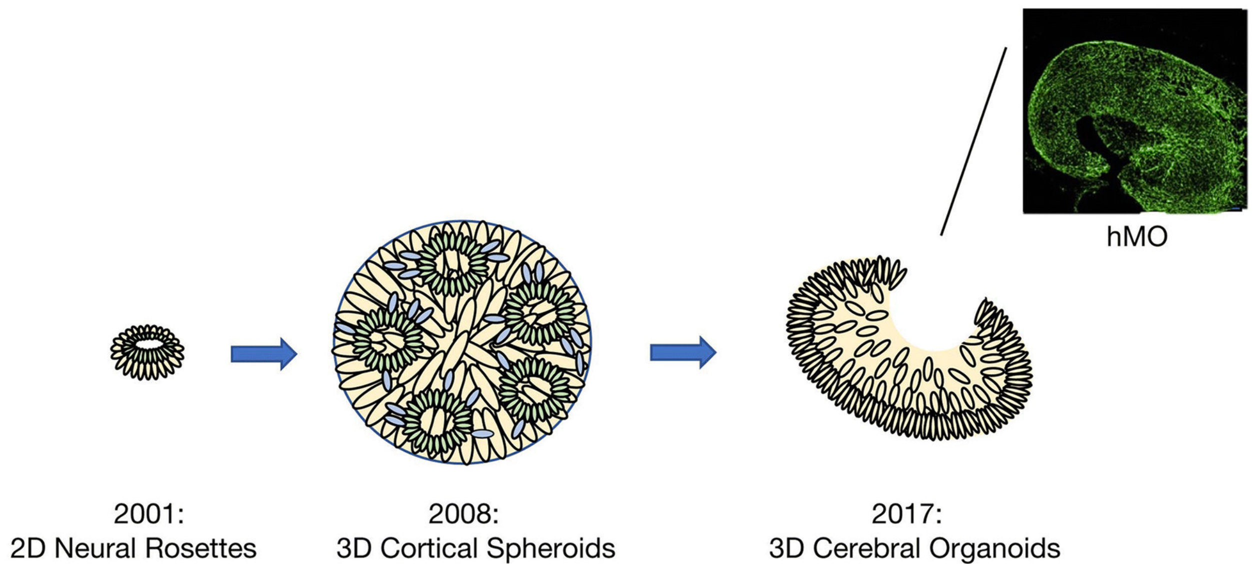

Figure 3. Neuronal Differentiation and Self-organizing in HMOs
Starting from the initial development of neural rosettes in 2001, developmental models of the brain have become more complex and representative of corticogenesis. In 2008, the development of cortical spheroids allowed for the modelling of both deep and superficial cortical neurons, which then self-organized in a manner that resembled early corticogenesis. More recently, human pluripotent stem cell (hPSC) derived cerebral organoids allow for self-organization and self-patterning of several brain regions. Cerebral organoids have also enabled the development of more specialized structures, such as human midbrain-specific organoids (hMOs). Immunostaining for midbrain dopaminergic neuron (mDN) markers TUJ1 (green) reveals clearly specified clusters of mDNs within hMOs[124]*. In the schematic, green represents neural stem cells, cream represents intermediate progenitors and blue represents migrating or mature neurons.

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Table 1.

Summary of PSC-derived 3D culture models.

Germ Layer	Organ	Cell Types Required	3D Model	Reference
Endoderm	Pancreas	Pancreatic progenitors	Pancreatic spheroids/organoids	20,23,200
	Liver	Hepatocyte progenitors, endothelial cells (HUVECs), hMSCs	Liver bud organoids	92
	Intestine	Intestinal progenitors	Intestinal organoids	16
	Stomach	Gastric progenitors	Gastric organoids	201
	Lung	Lung progenitors	Lung organoids	158,202
Mesoderm	Heart	Cardiomyocyte progenitors	Cardiospheres	99
	Kidney	Renal progenitors	Kidney/ureteric bud organoids	17,19,100
		Ureteric bud progenitors, mouse metanephric mesenchymal cells	Chimeric kidney organoid	203
	Blood	Megakaryocytes	Megakaryocyte spheroids	98
Ectoderm	Retina	Retinal progenitors	Optic cup organoid	14
	Brain	Neural progenitors	Cerebral organoids	10,18,91
		Neural progenitors	Midbrain organoids	204
		Neural progenitors	Neurospheres	6

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Table 2.

Summary of adult stem cell-derived 3D culture models

Organ	Isolated Cell Type	3D Model	Reference
Bone	Human articular chondrocytes (hACs), hMSCs	Chondrocyte spheroids	93
	Human osteoblasts	Bone spheroids	205
Small Intestine	Intestinal stem cells	Small intestinal organoids	15,40
Colon	Intestinal stem cells	Colonic organoids	15
Lung	Alveolar epithelial progenitor (AEP), human lung fibroblasts (CCL-171)	Lung organoids	41
Liver	Mouse liver cells	Liver organoids	37
Stomach	Mouse gastric stem cells	Gastric organoids	206
Pancreas	Mouse pancreatic progenitors	Pancreatic organoids	39
Prostate	Prostate stem cells	Prostate organoids	207
Ovary	Fallopian tube stem cells	Fallopian tube organoids	208

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Table 3.

Summary of existing in vitro 3D disease models

Organ	Associated diseases	Reference
Brain	Alzheimer's	150
	Neuropsychiatric diseases (autism, schizophrenia, bipolar disorder)	149
	Microcephaly	18
Kidney	Polycystic Kidney Disease, Fibrosis	163,165
	Nephronophthisis	164
Eye	Retinal Degeneration	153
	Glaucoma	152
Stomach	Infection with H. pylori	209
Pancreas	Cystic fibrosis	166
	Pancreatic Cancer	192
Liver	Hepatocellular carcinoma and cholangiocarcinoma	190
	Hepatitis B virus	162
Heart	Dilated cardiomyopathy	154
	Cardiac Hypertrophy and Heart Failure	107,155
	Cardiac sodium channel disease and related cardiac arrhythmias	157,210
Lungs	Influenza	160
	Congenital surfactant deficiency syndrome	158
	Asthma	211
	Pulmonary Edema	4
Multiple organs	Systemic toxicity	113,169