Stem Cell Biology and Regenerative Medicine

Badrul Hisham Yahaya *Editor*

Organoid Technology for Disease Modelling and Personalized Treatment

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Our understanding of stem cells has grown rapidly over the last decade. While the apparently tremendous therapeutic potential of stem cells has not yet been realized, their routine use in regeneration and restoration of tissue and organ function is greatly anticipated. To this end, many investigators continue to push the boundaries in areas such as the reprogramming, the stem cell niche, nanotechnology, biomimetics and 3D bioprinting, to name just a few. The objective of the volumes in the Stem Cell Biology and Regenerative Medicine series is to capture and consolidate these developments in a timely way. Each volume is thought-provoking in identifying problems, offering solutions, and providing ideas to excite further innovation in the stem cell and regenerative medicine fields.

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Contents

Chapter 1
The Organoids: Derivations and Applications

Ahmad Faried, Yulius Hermanto, Putri R. Amalia, and Hendrikus M. B. Bolly

Abstract Because of sample availability and ethical considerations, the biology of human tissues and organs is challenging to research. However, advancements in stem cell culture make it feasible to generate in vitro three-dimension (3D) tissue that exhibits some of the genuine organoids' main multicellular, anatomical, and even functional properties. Organoids offer a wide range of uses in fundamental research, drug discovery, and regenerative medicine since they may simulate organ development and illness. Although organoids have some shortcomings in their application, they hold great potential in the future for clinical applications. **Methods**: For the selection of literature cited, we used the Pubmed database. The keywords used in the MEDLINE research were: organoid, stem cells, disease modelling, 3D culturing. **Results**: Pluripotent stem cells [(embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs)], neonatal or adult stem/progenitor cells produced in vitro can be used to make organoids. Organoids can be used to stimulate development, homeostasis, regeneration, disease modelling, drug screening and testing, personalised medicine, and regenerative medicine, among other things. **Conclusion**: Organoids are 3D in vitro tissues with some of the major multicellular, anatomical, and even functional features of real organs, and because of these characteristics, they have been applied in various fields. Despite some drawbacks, organoids hold great potential in the future for further clinical applications.

Keywords Organoid · Stem cells · Disease modelling · 3D culturing

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Introduction

Initially, the theory of human organs' development and function was mostly speculation because most human tissues were inaccessible for research. This understanding of the development and function of human organs has only improved significantly in the last century [1]. This improvement is mainly due to discovering research subjects ranging from *Drosophila* fruit flies, *C. elegans* worms to research models of vertebrates such as mice and zebrafish [2]. Although these research models have brought about significant improvements, there are differences between animals and humans that cause studies' failure to develop effective therapies. Over the decades, developing ex vivo human models has been extremely difficult to deal with due to the accessibility of tissue samples and related ethical issues [2, 3]. Breakthroughs in stem cell culture have enabled the creation of in vitro 3D tissues known as organoids, which have many of the main multicellular, anatomical, and even functional characteristics of true organs [3]. As the name suggests, an organoid means a structure that resembles an organ. Organoids are composed of several organ-specific cell types, can recapitulate several organ-specific functions (e.g. excretion, filtration, neural activity, contraction), and are grouped and arranged spatially organised similar to an organ [4]. The term 'organoid' has a broad definition. It has been used to describe many forms of in vitro cultures, ranging from tissue explants to organ-on-chip systems [3]. Here, organoids are defined as 3D structures derived from pluripotent stem cells [(embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs], neonatal or adult stem/progenitor cells grown in vitro, in which the cell spontaneously organised itself into a properly differentiated functional cell type and recapitulates at least some organ function [2, 5, 6].

Deriving Organoids

The key aspects of organoid formation are self-assembly and differentiation [2]. It usually entails the self-organisation of a somewhat homogenous cell population [3]. Even in the presence of a homogeneous signalling environment, a cellular system that lacks an ordered structure can be spatially reorganised by system-autonomous mechanisms. Self-organisation is the process responsible for this. Conceptually, the process of self-organisation may be split into two parts: self-patterning events and morphogenetic rearrangements [7]. Self-patterning is described as the development of cell differentiation patterns in an originally homogenous system due to system-autonomous processes and intracellular communication [7, 8]. The interplay between several different mechanisms has been proposed, and it includes reaction-diffusion mechanisms [9], bistabilities of regulatory networks [10], and asymmetric cell division [7].

On the other hand, Morphogenetic rearrangement is the sorting of various cell types within tissue and the higher-level reorganisation of the system's architecture.

Differences in cell adhesion, cortical tension and/or contractility, and cell motility, which facilitates cell sorting, all play a role in the physical contact between various cell types [7, 11]. The system-intrinsic mechanics caused by cell shape changes, cell contraction, cell movement, or differential tissue expansion keeps architectural rearrangements in place [7]. The recapitulation of this process influenced the success in organoid derivation. Besides, it is also influenced by the physical characteristics of the cultural environment; requirements for system autonomous (i.e., endogenous) and/or exogenous signals; and initial cell types and system conditions, which will be explained in further following paragraphs.

Physical Properties of the Culture Environment

To promote the 3D characteristics of organoids, the solid extracellular matrix (ECM) that support cell growth and cell adherence can be used. The most widely used matrix for 3D organoid derivation is Matrigel, a natural ECM purified from Engelbreth-Holm-Swarm rat sarcoma [3]. Some examples of organoids that have been successfully made using Matrigel or similar animal-derived hydrogels that mimic the basement membrane include intestinal, cerebral [12], gastric [13], and mammary gland organoids [3]. Although rare, organoids derivation of the mammary glands and intestines can use a type I collagen matrix [14, 15]. This natural matrix's unique combination of ECM components and growth factors promotes effective cell development and differentiation. However, the diversity and complexity of these compositions makes controlling the cultural milieu challenging and reduces repeatability. To address this, a hydrogel was recently created to sustain intestinal and brain organoid cultures, allowing the metabolic and environmental processes of the culture to be regulated [16, 17]. But they are inherently less bioactive and need to be adapted to the specific requirements of different organoids.

One strategy used to generate optic cup [18], cerebral, cerebellar [19], and hippocampal organoids are the culture of 3D cell aggregates in suspension [20]. The suspension culture method does not employ solid scaffolding for cell embedding to encourage the development of polarised epithelial structures. Low quantities of Matrigel may be applied in some situations [3, 18].

Renal organoids may be created utilising the air-liquid interface technique, which involves the growth of cells in the form of pellets on a thin porous membrane with the cell culture medium only on the basal side of the membrane [21, 22]. The cell pellets then self-organise into a multilayered structure similar to the original kidney's microarchitecture.

The utilisation of particular organoid derivation techniques is currently mostly empirical. There is a dearth of systematic comparison of different procedures for obtaining certain organoids, making it impossible to understand each technique's relative strengths, limitations, and uses [3].

Endogenous and Exogenous Signals

Organoids are generated due to the appropriate developmental signalling pathway being activated and are mostly derived from an initial cell population exposed to certain morphogens at a specific moment in time. If all of the required components are present in the system, these signals can cause self-organisation. Exogenous provision of missing components is required [3].

Some organoids rely nearly completely on endogenous cues to develop. Mouse optic cup organoids generated from mouse PSCs, for example, were collected and grown in a serum-free medium with low growth factor levels. These circumstances promote the development of homogenous neuroepithelium (NE), after which a self-patterning mechanism determines the spatially distinct domains of the neural retina (NR) and retinal pigmented epithelium (RPE). Then, even if no external signal is supplied, morphogenesis can proceed since the starting cell population already has all the components required to arrange itself into an optic cup.

Although the mouse optic cup organoid is nearly entirely reliant on endogenous signalling, most organoid derivation procedures need the addition of particular exogenous signals since the original cellular system lacks all of the necessary components for the intended self-organisation process. In other situations, the exogenous signal is only necessary for the initial cell type to be induced and the remaining self-organising processes to be carried out using the system's autonomous signal. Human PSCs (hPSCs), for example, must be exogenously activated with particular growth factors to generate a mixed population of ureteric epithelial cells and metanephric mesenchyme. The cell population will then arrange themselves into kidney organoids without adding any additional substances to the medium [3, 21, 23].

Many organoid cellular systems, such as stomach organoids generated from hPSC, require stimulation by an appropriate and particular exogenous signal during the derivation process. An exogenously provided factor is necessary to drive definitive hPSC-derived endoderm cells to the posterior foregut destiny [13]. Exogenous stimulation is necessary to control the development, morphogenesis, and differentiation of the cells into functional gastric cell types and to guide them to form the antral or fundic gastric epithelium [13, 24].

Cell Sources, Starting Cell Type, and Initial Culture Condition

The cell source for organoid formation (Fig. 1.1) can be derived from primary tissue or differentiated from pluripotent stem cells, such as embryonic stem cells (ESC) or induced pluripotent stem cells (iPSCs) [3, 25]. iPSCs are easy to obtain and individual-specific. ESC and iPSC can differentiate into almost any type of body tissue [25]. When trying to mimic the complexity of native tissue, the heterogeneity of cell types produced in organoid cultures derived from pluripotent stem cells can be an advantage [24, 26, 27]. However, the unintended heterogeneity of the culture

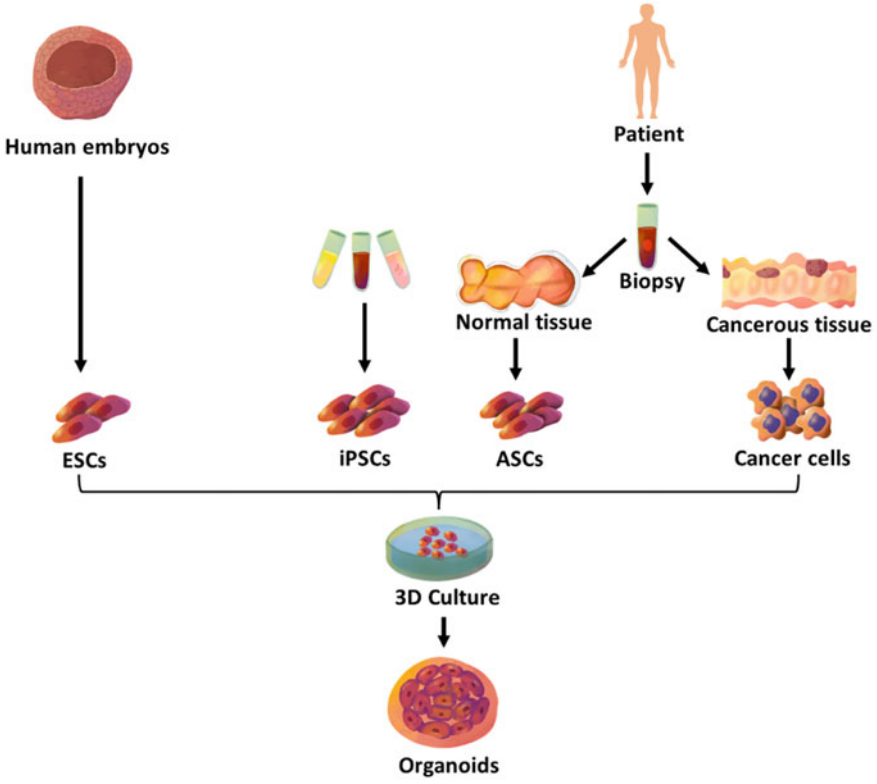

Fig. 1.1 The cell source for organoid derivations

of pluripotent stem cell strains and incomplete knowledge of specific differentiation signals can have unintended consequences for the resulting organoid [25]. This is shown in single-cell transcriptomic studies that iPSC-derived and ESC-derived kidney organoids comprise 10–20% of non-kidney cells, such as brain and muscle cells [28]. Also, organoids derived from pluripotent stem cells may exhibit a gene expression pattern more reminiscent of fetal tissue than from their adult counterparts [13, 29, 30].

In terms of the starting condition of the cell population, the methods utilised in the generation of distinct organoids differ. Depending on the starting circumstances of the cell population, some cells go through all of the self-organisation processes, while others just go through a subset of them. Self-organisation of the cell population requires symmetry-breaking and subsequent patterning to generate spatially distinct domains of the multiple cell types in organoids derived from a single cell type (such as the optic cup or small intestine organoids). The patterned structure is then morphogenetically rearranged to produce the final organoid architecture. In general, beginning from a single cell, organoid derivation methods need an initial stage of cell growth before self-organisation can occur [3]. Some methods call for co-culturing of

cell types that have been pre-differentiated independently (for example, PSC-derived liver organoids) [31]. This protocol has mainly established several cell identities. As a result, self-organisation mostly includes cell sorting and subsequent architectural rearrangements.

In addition, the starting circumstances of the cell population will have an impact on the use of organoids as a biological model system. Organoids created by co-culture of individually specified cell types, for example, are less instructive for understanding organogenesis than models in which diverse cell types are grown concurrently. As a result, it is more appropriate to examine the transitory developmental interactions that might occur between distinct progenitors during organoid creation [3]. The starting cell type also influences the characteristics of the final organoid produced. Organoids can be cultured from ASCs (either as isolated cells or from dissected tissue fragments), PSCs [12, 13], or fetal progenitor cells [32, 33]. Neuroectodermal organoids, such as the optic cup and cerebral organoids, and mesodermal renal organoids have been derived only from the PSC [3]. In contrast, organoids from surface ectoderm lineages (especially glandular tissue) are predominantly derived from ASCs or dissociated adult tissue [14, 34, 35]. Most of the endodermal lineage organoids originate from PSCs and ASCs [3].

Different cell types emerge at distinct phases of development and take different paths. As a result, while investigating the factors behind organoid development, choosing the starting cell population is critical. ASCs or adult tissue fragments cultured are thought to create organoids that replicate their original tissue's homeostatic or regenerative circumstances. Thus, stem cells derived from organs with a high renewal rate, such as the epithelium of the small intestine, colon [36] or stomach [37], generate organoids that mimic the homeostatic role of these cells in vivo. Organoids produced from slow turnover tissue, such as the pancreas or liver, in which endogenous stem cells and/or progenitors may play a role only the following damage, on the other hand, are regarded as genuine regeneration models [38, 39].

As previously stated, ASC-derived organoids can help researchers address concerns regarding the biology of adult tissues. PSC-derived organoids are primarily utilised to research organogenesis and tissue development [3]. PSC-derived organoids seldom reach the mature tissue stage in vitro. They usually resemble foetal tissue [13]. The restricted development of PSC-derived organoids is most likely owing to progression to more mature cell types, which necessitate continuous culture for a period of time that typically surpasses the capability of the actual culture methods [40]. The several organoids that may be produced from PSCs and the developmental signals [41] are shown in Fig. 1.2.

Next is the embryonic organoid system, which is also called 'embryoids' or 'gastruloids'. These organoids mimic in a very simple way pre-implantation [42] and early post-implantation embryo development [43–45], body axis formation [46, 47], gastrulation [46–52], and neural tube development [53, 54]. Unlike classical organoids, which usually consist of a limited subset of cell types from one germ layer, embryonic organoids contain cells from several germ layers, as in real embryos. These organoids can be used to build a complete development model in vitro and

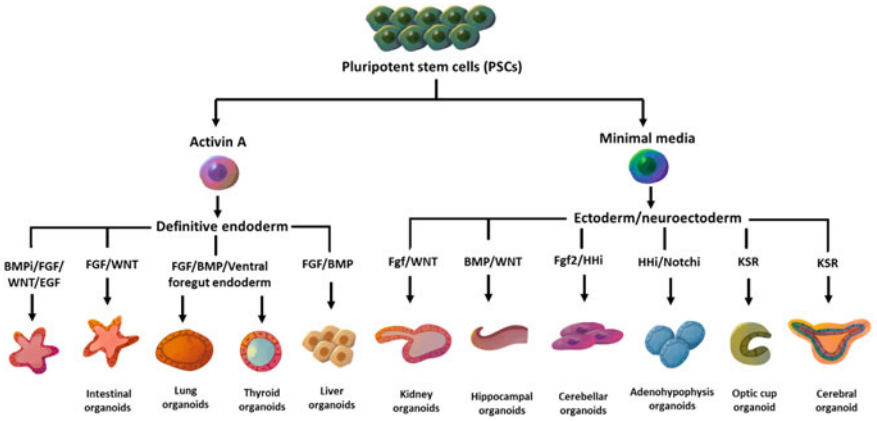

Fig. 1.2 Various organoids that can be grown from PSCs and the developmental signals that are used (Reproduced from [41])

to study the complex interactions between different cell types in the development process [3].

Finally, organoids start directly from the fetal tissue (between the ASC and PSC stages), fetal progenitor-derived organoids [32, 33]. Compared to PSC-derived organoids, fetal organoids can be used to study advanced organogenesis, for example, as has been done to study the enterosphere maturation of fetal intestinal progenitors [32].

Applications of Organoids

The Use of Organoids as Models for Development, Homeostasis, and Regeneration

In fundamental research, organoids can be utilised to better understand development, homeostasis, and regeneration principles. As simplified and conveniently accessible' minimal systems,' Organoids can recreate in vitro some organ biology principles and differentiate the relative contributions of distinct tissue components to complicated morphogenetic processes [3]. Organoids have helped to better understand organogenesis, human development, and adult organ biology due to their ease of access. Organoid cultures can be used to investigate the similarities and differences in the development of humans and other animals. This is critical for understanding human brain development and congenital disorders [2].

The Use of Organoids for Disease Modelling

One of the great potential applications of the organoid model is to analyse human-specific disease mechanisms [2]. Compared to the traditional cell culture of the single-cell type, organoid culture as a disease model can mimic pathology at the organ level [3]. Organoids have been used for modelling various diseases such as genetic diseases [12, 55, 56], diseases involving host-pathogen interactions, and even cancer. This proves that organoids are capable of reproducing certain well-known pathological features. For example, microinjection of the bacterium *Helicobacter pylori* into human gastric organoids reproduces the typical signs of this bacterial infection [13, 37]. This model is particularly relevant because species-specific gastric characteristics make animal models unsuitable for studying the pathology of the human stomach. H. pylori infection in mice does not develop into ulceration and cancer as in humans [37]. In essence, organoids have been used to study congenital and acquired diseases. The following are some of the diseases that have been studied using organoids.

Congenital Diseases

The first human disease to be mimicked using organoids was cystic fibrosis (CF). Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel, usually expressed on the epithelium of numerous organs, cause this disease [57]. Surface expression of CFTR was missing in iPS-derived lung organoids from CF patients to replicate the in vivo condition in CF [58]. Dekkers and colleagues developed intestinal organoids from CF patients that may imitate the disease in vitro. They developed a swelling experiment in which wild-type organoids respond to cAMP stimulation by importing fluid into the lumen and swelling, but CF organoids do not [55]. This technique is effective for detecting responders to CFTR modulators and has a high predictive value. The Verma lab generated iPS cells from CF patients and utilised CRISPR/Cas9 to repair the mutation. The repaired iPS cells were subsequently converted into mature airway epithelial cells, exhibiting normal CFTR function [59].

Primary microcephaly, a genetic disorder induced by CDK5RAP2 mutations, is another congenital ailment investigated with organoids [12]. The brain organoids derived from patient-derived iPSCs were much smaller and the individual cortical regions were primarily hypoplastic. A series of observations and specific examinations of the orientation of the mitotic spindle during progenitor division revealed that the patient's neural stem cells began to divide asymmetrically and generate neurons prematurely, leading to depletion of the progenitor pool ultimately the decline of overall neurons. Because mice could not properly reproduce the amount of brain shrinkage found in humans, the organoids showed morphological abnormalities that could only be detected in this human-specific model system [57]. Organoids can also

be used to simulate idiopathic autism spectrum disease (ASD). Mariani et al. generated iPSC lines from four autistic individuals and four unaffected controls. These were produced first as 3D aggregates, then rosettes were plated. The rosettes were then separated and regrown as 3D aggregates to produce forebrain organoids [60, 61]. Although probands and controls were usually fairly comparable, the ASD organoids had more inhibitory interneurons due to elevated FoxG1, an essential component in forebrain patterning [57].

Acquired Diseases

Apart from modelling the inherited conditions of patient stem cells carrying genetic mutations, organoids can also be used to model acquired diseases such as acquired mutations as in the case of cancer and diseases caused by infectious agents. Organoids can be used to model lung [62], stomach [37, 63, 64], liver [65, 66], pancreatic [66–68], colon [36, 63], Van [69], prostate [70], endometrial [71], breast [72], bladder [73, 74], esophageal [36, 75], and brain cancers [76]. These organoids come from tissue resection, biopsy or even circulating tumor cells. Cancer-derived organoids are more likely to retain the genetic and phenotypic features of the original tumor. In this respect, they resemble patient-derived xenografts, but have the advantages of a higher success rate, can be easily expanded in vitro, and can be used for drug screening [77, 78].

Organoids have shown to be a useful model for investigating infectious illnesses and the processes underlying human-specific infectious agents [57]. Models of Helicobacter pylori infection have been developed using gastric organoids [13, 37], whereas influenza virus infection has been mimicked in vitro using pulmonary organoids [79]. Human intestinal organoids can be used to spread coronaviruses in vitro and have enabled the identification of the small intestine as an alternative infection pathway for Middle East respiratory syndrome (MERS) coronaviruses, which cause severe human respiratory infections [80]. With the recent outbreak of the COVID-19 pandemic, substantial efforts have been undertaken to simulate and understand the biology and pathophysiology of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection [81]. Several investigations utilising organoid models generated from ASCs found that SARS-Cov-2 may infect enterocytes [82], and they revealed that viral replication in enterocytes resulted in viral response gene upregulation and the production of infectious viral particles. In human small intestine enteroids, two mucosa-specific serine proteases, TMPRSS2 and TMPRSS4, facilitate viral entrance and infection of enterocytes [83].

The Use of Organoids in Drug Discovery and Personalised Medicine

Disease-specific organoids are useful in identifying new biomarkers, personalise drug testing, drug screening [3], or toxicology studies, and thus organoids will turn personalised medicine into reality [2]. One of the uses of organoids for drug testing that has been carried out is drug screening for the therapy of Zika virus infection using cortical nerve progenitor cells derived from hPSC and validated parallel in organoid and mouse models [84]. Organoids have also been used in testing drugs for cystic fibrosis (CF), a genetic disease caused by defects in the CFTR gene. Intestinal organoids derived from cystic fibrosis patients who carry CFTR mutations are an example of organoids in personalised medicine [3, 85]. To find out whether existing CF drugs can give a good response, screening is carried out using organoids from patients with CFTR mutations to get the appropriate treatment [86].

Personalised medicine using organoids can also be applied in drug testing for cancer. Organoids derived from various human tumours have demonstrated a response spectrum of conventional and investigational drugs to date [87]. Based on a retrospective cohort study, the patient-derived organoid (PDO) response to tested therapy largely mimicked the initial response of these patients to the same agent [73, 77, 88], [89] [90]. PDO also provides a model for drug development without innate or acquired resistance, and it is particularly relevant in ovarian cancer PDOs in the assessment of DNA repair pathways and the stability of the replication fork [88]. Besides, PDO may also reflect a patient's clinical response to a cytotoxic drug having a narrow therapeutic index in vivo compared to many targeted agents by demonstrating a relative sensitivity response to the cytotoxic drug [77] [89] [90].

Moreover, the creation of organoid biobanks for various pathologies is also very promising. The organoid biobanks will facilitate a robust screening platform that covers a wide variety of population genetics worldwide. With this organoid biobank, most of the spectrum of CFTR mutations in cystic fibrosis and other diseases can be covered [3]. Especially for cancer, a disease with a virtually unlimited number of mutations, making this organoid biobank can be very useful [69, 91]. The creation of cancer organoids can use neoplastic tissue directly or by using normal tissue, which is then genetically modified [87]. Early attempts to create a tumouroid biobank have been made for colon cancer, a very common cancer in humans [69, 91]. In the end, the use of organoids can reduce the experimental animal for research which is following the 3R principle.

The Use of Organoids in Regenerative Medicine

Organoids are a promising alternative in regenerative medicine [3]. Organoids that have the potential to produce human 3D cultures that resemble specific organs have opened up the possibility of using organoids as a source of cell therapy and as an

alternative to whole organ transplants [2]. This concept has been proven through experiments in animal models. An example is the transplantation of retinal sheets derived from mouse embryonic stem cells (ESCs) or mouse iPSCs using a modified optical cup organoid protocol in mice with retinal degeneration. The transplanted tissue can give rise to mature photoreceptors and, in some cases, capable of forging synaptic relationships with host cells [92] and restoring light responses [93]. The same was observed in the retinal tissue produced from organoids cultured from human ESCs. When transplanted into mouse and monkey models with retinal degeneration, the resultant tissue can survive, develop, and integrate with the host tissue to some extent [94]. Intestinal organoids derived from dissociated mouse colon epithelium or single stem cells implanted into mice, for example, can repair colonic mucosal damage to various degrees [95]. Animal models have also been used for liver [39, 56] and kidney [96] organoids transplant studies.

Also, organoids can be combined with novel genome-editing tools such as CRISPR/Cas9 to correct affected genes and select appropriate clones before autologous transplantation [2, 3]. CRISPR-Cas9-mediated gene editing was used to correct the most common CFTR mutation in CF. Phenylalanine removal at position 508 on the ISC derived from the patient was then used to produce functional organoids [97]. Although autologous cell therapy transplantation is very promising in the field of organoids, its efficacy, safety, and immunogenicity are still being evaluated [2].

Challenges, Limitations in the Application of Organoids, and Bioengineering Approach to Overcome Limitations

The previously described organoids application is based on creating repeatable organoids that are structurally and functionally comparable to actual organs and may be utilised as appropriate replacements for in vivo research. The primary issue over the next few decades will be bridging the gap with native organs. A frequent drawback is the considerable phenotypic heterogeneity that can occur from all organoid generation procedures. The constraint in many applications is organoid-to-organoid repeatability. This is especially true for translational investigations, such as drug screening, where significant inherent variability may obscure treatment impact. Furthermore, the cellular and architectural intricacy of each organ was reproduced with varying degrees of precision. This is referred to as the organoid capacity to generate all sorts of cells in a certain tissue as well as multiple organ tissues [3]. Intestinal organoids produced from ASCs, for example, are entirely comprised of the intestinal epithelium; nevertheless, intestinal mesenchyme can also emerge from the derivation of intestinal organoids utilising PSCs [30]. Another important limitation is the low level of maturity, especially for PSC-derived organoids, thus hindering their application as a model for adult tissue biology [3]. Other disciplines can help overcome these limitations and will be discussed in the following paragraphs.

Approaches to Improve Organoid Maturity

The limited maturation of cells is the major drawback of PSC-derived organoids. Usually, this type of organoid resembles fetal tissue more than adult tissue [13]. The limited lifespan of organoids can be one reason limiting their ability to reach the next stage of development [3]. The limited lifespan of the organoids can be caused because diffusion cannot supply all cells with sufficient nutrients to support their continued development once a certain size is reached [98]. The use of bioreactors that increase nutrient supply through constant culture spinning is a possible solution to this problem. In tissue engineering, bioreactors are widely used to introduce controlled change in culture conditions, standardisation, and enhancement of tissue production for regenerative medicine [99]. Such bioreactors have successfully extended the duration of brain organoid cultures from several months to 1 year and produce structures more similar to those of the developing human brain [6].

Furthermore, to increase the lifespan of organoids, artificial vascular tissue can also be used. Organoid vascularisation can distribute nutrients via capillaries, as occurs in vivo [3]. Bioengineering approaches have been developed to produce vascular tissue-like structures, namely *sacrificial molds* [100–102] and *laser ablation* [103], which allow for creating channels in culture scaffolds that can accommodate endothelial cells and form vascular units that can be perfused. An alternative bioprinting method can be used to control the position of endothelial cells in a 3D printed structure [104, 105]. Another technique used to improve the vascularity of developing tissue is to incorporate endothelial cells or their progenitors during organoid development. This method has been used effectively with liver organoids. Human endothelial cells are grown with human mesenchymal stem cells and liver endoderm cells generated from human iPSCs in this technique to form self-organising liver buds with a microvascular network linked to host circulation soon after transplantation into mice [31].

Another possibility of limited organoid maturation is the lack of specific factors in the in vitro environment, so it cannot reach the expected maturation level [3]. For example, sensory stimulation of brain organoids is needed for further maturation to occur. This sensory stimulation contributes to the formation of neural circuits in vivo. For human organoids, a longer culture time is required because it takes longer to mature than mouse organoids at the same stage [18, 106].

Approaches to Improve Organoid Architecture

The organoid 3D microanatomy produced through self-organisation does resemble an in vivo organ, but the overall architecture is different from the actual organ. A stem cell culture scaffold with tissue-appropriate topography can be used to overcome this so that the organoid architecture can be improved and the size can be increased [3]. Micro-collagen gels, for example, that replicate the unique crypt structure of the

colonic epithelium have been utilised to cultivate a single layer of self-renewing human colonic cells [107]. Organoid topology can be improved at the microscale by precisely controlling the stem cell interactions and the surrounding ECM [3]. Matrigel, a naturally generated hydrogel matrix, is employed in the majority of the matrices. Because they are ill-defined, have lot-to-lot variability, and do not enable controlled alteration, these matrices are ineffective for directing organoid morphogenesis, despite being very successful at stimulating cell proliferation and self-organisation. This matrix also includes animal-derived goods, which are unsuitable for clinical usage due to the danger of transmitting immunogens and infections [3]. Several synthetic and chemical hydrogels for 3D cell growth have been created to address these constraints, with chemical and physical characteristics that may be manipulated and tuned for specific uses [108, 109]. Cerebral organoids, for example, have been implanted effectively in hyaluronan-based hydrogels [17], and neural tube cysts have been produced in poly (ethylene glycol) (PEG)-based hydrogels [53, 54]. Recently, new hydrogel formulations with spatially and temporally modulable biochemical and biophysical characteristics have been created [16, 110]. Controlling how cells combine might potentially be used to expand control over the organoid's self-organisation. Positioning distinct cell types in conformations that skew cell-type-specific spatial interactions, for example. Furthermore, by controlling diffuse signalling molecules' geographical and temporal distribution, organoid development may be accelerated [3].

Approaches to Improve Disease Modelling

The primary drawback of organoids in disease modelling applications is their inability to simulate multi-organ diseases. A co-culture approach can help to alleviate some of this [3]. Intestinal organoids and hPSC-derived enteric neurons were used in early attempts in this approach [111]. Furthermore, the present drug testing platform may be improved by merging organoid cultures with organ-on-chip technology to build a 3D system that mimics the interaction between multiple organs. With this technique, the advantages of both systems (the conventional basic organ-on-chip technology and the high in vivo fidelity and functionality of organoids) may be merged [3].

Conclusion

Organoids are 3D in vitro tissues that exhibit some of the key multicellular, anatomical, and even functional properties of actual organs, and they have been used in a variety of disciplines due to these qualities. Despite certain limitations, organoids have much potential for future therapeutic uses.

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Chapter 2
Lung Organoids: A New Pathway into Lung Regeneration and Repair

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Abstract **Introduction**: The lung is an important and complicated organ that has profound impacts on the entire body when impacted by disease or illness. Due to its complexity, the human lung is difficult to study; lung models that can mimic this organ are key to better understanding and treating lung diseases. Various lung models have been developed over the years, but one important and recent model is the lung organoid model. Here we review human lung organoid models, including the main characteristics and potentials and their current and future applications for modelling lung development and diseases. **Method**: For the selection of literature cited, we used MEDLINE/Pubmed database. The keywords used in the MEDLINE research were: human lung development, lung organoids, lung stem cells, lung disease and repair, bioengineering lung. **Results**: Lung organoids, in layman's terms referred to as "mini lungs in a dish", are 3D tissues that recapitulate the endogenous functions of the lungs. Lung organoids currently represent the closest model to the human pulmonary system. Human-derived three-dimensional (3D) models have been generated, allowing for a deeper understanding of cell-to-cell communication. They have also allowed researchers to better understand how diseases affect the lungs and determine potential treatment methods. **Conclusions**: Although the area of research using lung organoids is still relatively new, much has been learned from this model, and

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much more will continue to be learned. There is an urgent need to develop more complex organoid models containing mesenchymal tissues and vasculature to better understand lung diseases.

Keywords Lung · Organoid · Stem cell · Repair and regeneration · Lung diseases

Abbreviations

PNECs	Pulmonary neuroendocrine cells
AT1	Alveolar epithelial type 1
AT2	Alveolar epithelial type 2
AT2-s	AT2 signaling
PAECs	Pulmonary artery endothelial cells
PVECs	Pulmonary vein endothelial cells
aCap	Aerocyte capillary
gCap	General capillary
BAECs	Bronchial artery endothelial cells
BMVECs	Bronchial microvascular endothelial cells
ARDS	Acute respiratory distress syndrome
ALI	Acute lung injury
E	Embryonic
P	Postnatal day
pcw	Post-conception weeks
SMCs	Smooth muscle cells
hPSCs	Human pluripotent stem cells
BME	Basement membrane extract
h3AC	Human 3D alveolar type 2 cell culture
hAT2	Human AT2
ESCs	Embryonic stem cells
iPSCs	Induced pluripotent stem cells
DE	Definitive endoderm
AFE	Anterior foregut endoderm
BMP	Blocking bone morphogenetic protein
TGFβ	Transforming growth factor beta
FGF	Fibroblast growth factor
RA	Retinoic acid
HH	Hedgehog
LBOs	Lung bud organoids
iAT2s	Isolated alveolar epithelial type 2 cells
SFTPC	Surfactant protein C
pro-SFTPC	Pro-surfactant protein C
HLOs	Human lung organoids
ASCsyy	Adult stem cells

CF	Cystic fibrosis
IPF	Idiopathic pulmonary fibrosis
CFTR	Cystic fibrosis transmembrane regulator
HPS	Hermansky-Pudlak syndrome
HPSIP	HPS-associated interstitial pneumonia
GRHL2	Grainyhead-like 2
Lgr	Leucine-rich repeat-containing G protein-coupled receptor
DATPs	Damage-associated transient progenitors
ILDs	Interstitial lung diseases
COPD	Chronic obstructive pulmonary disease
GCM	Goblet cell metaplasia
RSV	Respiratory syncytial virus
HPIV3	Human parainfluenza virus type 3
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
ECM	Extracellular matrix
PEGDA	Poly (ethylene glycol) diacrylate
LADC	Lung adenocarcinoma
CDH	Congenital diaphragmatic hernia
PCD	Primary ciliary dyskinesia
SB	SB-431542
CHIR	CHIR99021
GSK	Glycogen synthase kinase
SAG	Smoothened agonist
SU	SU5402
MCC	Mucociliary clearance
SARS	Severe acute respiratory syndrome
MERS	Middle East respiratory syndrome

Lung Development, Homeostasis and Regeneration

Different Cell Populations in Human Lungs and their Role in Lung Development

Introduction

The lung contains a wide variety of cell types, many of which are still poorly understood or yet to be discovered. Various lung researchers distinguish these cells differently, and there is no universal method of cell distinction, but this chapter will focus on the current research confirming 58 cell types in the human lung. These cell types are placed into four different categories: epithelial, endothelial, stromal, and immune cells [1, 2].

Epithelial Cells

Epithelial cells make up most of the body tissues, which line the internal and external surfaces of the body [3]. There are 15 known epithelial cells in the lung. Twelve of these cells reside in the airway, and three reside in the alveoli. The major epithelial airway cell types are ciliated, undifferentiated columnar, basal, and secretory cells [2, 4, 5]. The airway epithelium plays a pivotal role in the first line of defence against unwanted particulates and pathogens. It also plays a role in the maintenance of funnelling air into the airways to facilitate gas exchange [4, 6].

Within the 12 airway epithelial cell types, there are morphologically distinct types with different functions [6]. Columnar ciliated epithelial cells possess cilia, which aids in transporting mucus away from the lung into the throat [7]. Mucus cells, also known as goblet cells, secrete mucin and create a mucus layer necessary for trapping harmful substances in the airway lumen for secretion out of the lungs [8]. Serous cells, also known as secretory cells, perform various functions with the secretions they produce. These secretions keep the mucosa lining moist, humidify inhaled air, and clean the inhaled air from unwanted particulates and organisms [9]. Basal cells are stem cells capable of differentiating into the mucus and ciliated epithelial cells [6]. They help the attachment of columnar epithelium to the basal lamina by providing an area for cell-to-cell attachment [10]. Club cells are similar to basal cells in that they also stem cells and can give rise to ciliated and secretory cells [6]. Club cells produce surfactant proteins A, B, and D as well as metabolize xenobiotic compounds [11]. Pulmonary neuroendocrine cells (PNECs) are a rare type of epithelial cell that secrete biogenic amines and peptides, which play a major role in lung development and airway function [6]. Another group of rare cells in the human lung are known as ionocytes; these cells have a high expression of FoxI1 and the cystic fibrosis transmembrane regulator (CFTR), which may play a role in respiratory diseases such as cystic fibrosis (CF) [12].

The alveolus is where gas exchange occurs and contains epithelial cells that aid in maintaining lung homeostasis [13]. These cells are alveolar epithelial type 1 (AT1), alveolar epithelial type 2 (AT2), and AT2 signalling (AT2-s) cells [2]. AT1 cells occupy 96% of the surface of the alveoli and, although take up a huge portion of the surface, are extremely thin in order to allow passive gas diffusion [14–16]. AT2 cells synthesize surfactants to prevent the lungs from collapsing and differentiate into AT1 cells to facilitate lung repair and maintain homeostasis [16, 17].

Endothelial Cells

Endothelial cells are key regulators of vascular homeostasis via the inhibition of coagulation of the blood and accommodate blood flow levels within the lung [18]. These cells also enable efficient gas exchange in the lung by ventilation-perfusion matching [19]. The human lung contains nine endothelial cells and is placed into five different categories: artery, vein, capillary, bronchial vessel, and lymphatic cell [2].

Pulmonary artery endothelial cells (PAECs) and pulmonary vein endothelial cells (PVECs) have similar functions but are two distinct cells [20]. Both cells have calcium entry pathways and respond to inflammatory stimuli but at different rates [20]. There

are three types of capillary cells within the human lung: aerocyte (aCap), intermediate, and general capillary (gCap) cells [2, 21]. The main function of all capillary cells of the lung is to perform leukocyte trafficking but each type of capillary cell expresses different genes that regulate this process [21]. Capillary cells have roles in hemostasis and lipid metabolism due to their production of pro-/anti-coagulants as well as fatty acids, respectively [21]. Bronchial artery endothelial cells (BAECs) and bronchial microvascular endothelial cells (BMVECs) play key roles in protein transudation [22]. Lymphatic cells contribute to alveolar clearance for greater efficiency in respiration [23]. These cells achieve this due to their close proximity to major airways and blood vessels involved in the process of respiration.

Stromal Cells

The lung contains nine different types of stromal cells [2]. Stromal cells are non-specific stem cells with the ability to become different cell types such as chondrocytes, osteoblasts, and adipocytes in order to replace old cells and aid in repair [24, 25]. Stromal cells also play a role in inflammation control and have been found in premature infants with lung issues such as acute respiratory distress syndrome (ARDS) [26]. Their prevalence suggests that these cells take part in lung regeneration, repair, and development.

Immune Cells

In the lung, there are 25 immune cell populations with various functions [2]. All of these cells are important for healthy lungs but some of the most important are neutrophils, macrophages, and lymphocytes which protect the lung by eliciting an immune response geared to remove and destroy unwanted pathogens [27].

Neutrophils, also known as polymorphonuclear leukocytes, are producers of highly reactive oxygen radicals that have been found to be involved in lung diseases such as ARDS and acute lung injury (ALI) [28, 29]. They are some of the first leukocytes to be activated by lung infection [29, 30]. Activated neutrophils phagocytose pathogens, resolve inflammation, clear damaged neutrophils, and modulate immune responses via cytokine release [28–30]. Macrophages work alongside neutrophils by clearing the dead or dying neutrophils as well as pathogens [30, 31]. They also release a wave of cytokines and chemokines to induce a rapid immune response by recruiting other cells [30, 31].

There are various subpopulations of lymphocytes, each with their own specialized functions, but their shared characteristics are their recruitment into the lung during infection, their involvement in the immune response via inhibition or recruitment of other cell types, and their production of anti-inflammatory signals [32, 33].

Cell Population Roles in Lung Development

Lung development is divided into five stages: embryonic, pseudoglandular, canalicular, saccular, and alveolarization [34]. A general overview of lung development is illustrated in Fig. 2.1. Within each stage, cells within the lung have specific functions that help progress lung development. Most research on lung development has been through studying mice; therefore, the following sections will briefly discuss mouse

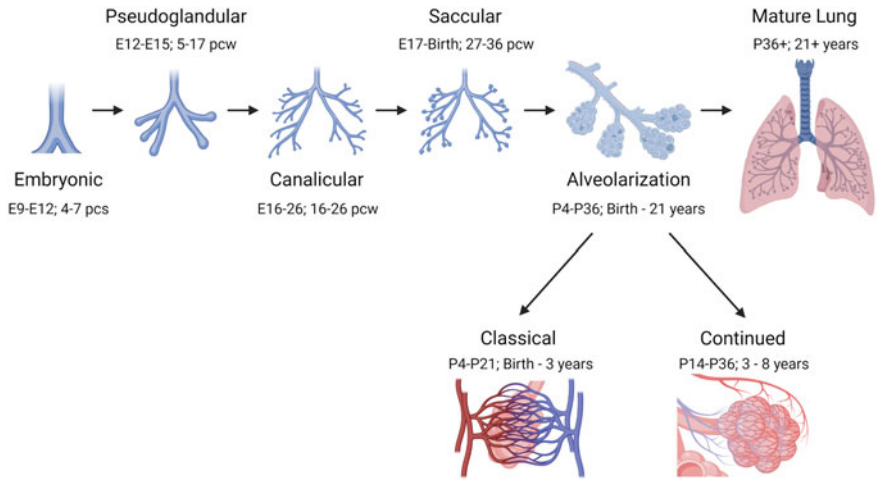

Fig. 2.1 Overview of lung development. E = embryonic; P = postnatal day; pcw = post-conception weeks. The developmental periods are placed in order of mouse lung development; human lung development. Classical alveolarization illustrates how the primary septae contain a double capillary network.

lung development and how it contrasts human lung development. The developmental periods beginning with embryonic (E) or postnatal day (P) will refer to mouse lung development stages and the developmental periods ending in post-conception weeks (pcw) will refer to human lung development.

Embryonic: E9-E12; 4–7 pcw

The development of the lung begins in the anterior foregut endoderm, which generates the respiratory endoderm [35]. The respiratory endoderm is what generates progenitor cells within the lung and begins on embryonic day 9.0 [34]. These progenitor cells, which are detected by the expression of Nkx2.1, then form the basic structure of the trachea and two lung buds that will form the left and right lobes of the lung [36]. The beginning of elongation of these lung buds into the mesenchyme is known as branching morphogenesis [5, 34]. The proximal progenitors generate PNECs, secretory cells, and goblet cells, whereas the distal progenitors produce AT1 and AT2 cells [34].

Pseudoglandular: E12-E15; 5–17 pcw

This stage begins when the bronchial tree is in the shape of a tubular gland and the epithelial tubules continue branching morphogenesis [5]. This is where maximal branching occurs [37]. The first 20 generations of the airway form by the end of this stage with primitive alveolar ducts [5, 38]. Columnar epithelial cells are found in the proximal airway and cuboidal epithelial cells are found in the distal airway [39]. The cells in the proximal airway differentiate into ciliated, non-ciliated, goblet, and basal cells. By the end of this stage, club cells are found in the trachea. Cells in the

distal airway remain undifferentiated until branching morphogenesis is complete, occurring as late as the saccular stage in the human lung [39]. Undifferentiated Sox2/Sox9 double-positive cells are found in the distal epithelial tips in the human lung. The surrounding smooth muscle cells (SMCs) in the distal lung appear to play a role in branching morphogenesis due to recent studies showing a decrease in Sox2+/Sox9+ cells coinciding with decreased branching in fetal lung explants treated with a toxin [37]. This does not occur in mouse lung development since the proximal and distal lung cells are already expressing different Sox transcription factors in the pseudoglandular stage [37].

Canalicular: E16-E26; 16–26 pcw

The canalicular stage is the beginning of the development of alveolar sacs [5, 38]. In this stage, the Sox2+/Sox9+ cells are no longer present in human lungs due to the surrounding SMCs suppressing the Sox9+ cell population in the proximal region [37]. At this stage, human lung development mirrors mouse pseudo glandular lung development in that the proximal progenitors express Sox2+ cells and distal progenitors express Sox9+ cells [37]. The cuboidal epithelial cells differentiate into AT1 and AT2 cells [5]. The air-blood barrier forms with the help of the endothelium of the capillaries coming into contact with AT1 cells [5]. AT2 cells begin to produce surfactant as well as differentiate into AT1 cells [5, 36].

Saccular: E17-birth; 27–36 pcw

Branching morphogenesis stops in this stage and is the transitional stage into alveolarization [5]. This intermediate stage is when the distal branches narrow and form small saccules (primary septae), which become alveoli (secondary septae) in the alveolarization stage [5, 34]. The alveoli begin to grow, widen, and form [5]. The primary septa is covered by predominantly AT1 cells with some AT2 cells filling in the space. Smooth muscle cells begin to form a network of elastic fibres and collagen fibrils. This network allows for the development of the alveoli by providing a scaffold for continued lung maturation [5]. Mice are born during the saccular stage and their lungs continue into the alveolarization stage after birth [37]. This differs from humans: humans are born in the alveolarization stage. Both mouse and human lung development continue after birth with the maturation of alveoli and the alveolarization stage for both species are similar [37]. The following explanation of alveolarization focuses on human lung development, but the main processes that occur during these stages are nearly identical in mouse and human lung development.

Alveolarization: P4-P36; human birth- ~3–15 years

Alveolarization is the process by which primary septae become secondary septae [5, 40]. Alveolarization is separated into two stages: classical and continued alveolarization [5]. Human classical alveolarization ranges from birth to about three years and human continued alveolarization ranges from around two years of age to young adulthood, estimated to be between 15 and 21 years [5]. Previous research believed that human lung development concluded at around 8 years of age [41] but new studies have shown that the number of alveoli continues to develop around 15 years of age,

with some subjects showing alveoli development into young adulthood (21 years) [42].

Classical Alveolarization: P4-P21; human birth-3 years.

The primary septae contain a double capillary network which is immature and inefficient for gas exchange. This stage is where the secondary septa are formed by the upfolding of one of the double capillary networks, resulting in secondary septae with a single capillary layer [5, 34]. This single layer formation, a process known as microvascular maturation, allows for the formation of alveoli [43, 44].

Continued Alveolarization: P14-P36; 3–21 years.

In continued alveolarization, microvascular maturation as well as classical alveolarization persist within the secondary septae, a process known as angiogenesis [5, 34, 43, 45]. This process moves distally over time and the alveoli mature as the child continues to grow. As the child gets older, alveolarization slows down.

The Challenge of Lung Modelling

The Complexity of the Human Lung

The lung is an extremely complex organ. Even with decades of research, researchers are still uncovering new knowledge about how the human lung functions.

Lack of Lung Tissue

There is limited access to lung tissue, especially fetal tissue, which is crucial to better understanding lung development. Most countries do not allow the use of human fetal tissue beyond 20 pcws, limiting the research on later lung development [46]. Later stages are typically studied using animal models, but various differences between animal and human lungs limits what researchers can learn about the human lung.

Differences Between Animal Models and the Human Lung

The types of animals used as a substitute for human tissues are rats, rabbits, mice, and rhesus monkeys [47−50]. There are various and significant differences between these animal models mentioned and human lungs. This is not meant to discredit the research performed on these animal models, but it is important to recognize that these studies have their limitations. Scientists have looked for other options for better understanding the human lung. The creation and use of lung organoids have become a practical option for researchers to study the human lung. Lung organoids remove two key issues in this field of research: the difficulty in obtaining human fetal lung tissue as well as the contrasting lung organization and cellular composition with the use of animal models.

Origin of Lung Organoid Cultures

Several groups have attempted to generate human lung organoids that can recapitulate essential features of human lungs in vitro. Generation of lung organoids usually includes endoderm induction, anterior-posterior patterning, lung specification, lung budding, branching morphogenesis, and maturation [51]. Currently, most lung organoids are developed either from human pluripotent stem cells (hPSCs) or stem cells isolated from primary tissues (Fig. 2.2). The resulting 3D human lung organoids are able to recapitulate various cell types, structures and some functions of mammalian lungs.

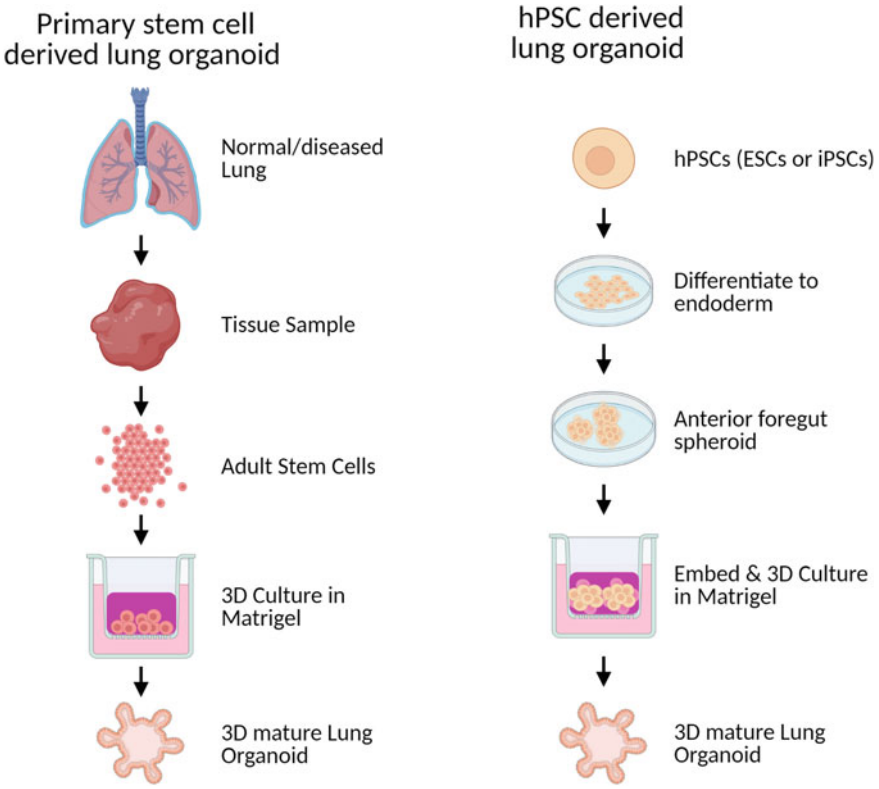

Fig. 2.2 Overview of the generation of primary stem cell- and hPSC-derived lung organoids. Primary stem cells used for the generation of lung organoids are obtained from normal or diseased lung biopsies. Tissues are processed into a single cell suspension and then cultured in Matrigel to expand and form 3D organoid structures. hPSC-derived organoids are differentiated and developed from either ESCs or iPSCs. After differentiating into definitive endoderm and forming anterior foregut spheroids by modulating various signalling pathways, the cells can be embedded into Matrigel to further branch and form 3D lung organoids.

Lung Organoids Generated from Primary Stem Cells

Several groups have made great efforts to generate human lung organoids from adult stem cells or fetal stem cells. These primary stem cells have the capability of self-renewal and differentiation into multiple cell lineages, which have great potential in forming 3D lung organoids. 3D spheroid/organoid structures can be formed from lung progenitors including basal cells [52] and AT2 cells [17].

Hild and Jaffe described a method to generate 3D airway organoids from primary human airway basal cells [53]. Commercially available human bronchial epithelial cells are used as basal cells and made into a suspension in 5% Matrigel. The cell density is made to be 30,000 cells/mL and 20 μl are seeded onto each well of a Matrigel-coated 384-well plate to have 600 cells/well. Matrigel is added to each well on day 2 (48 h after incubation) and again on day 8. Lumen are observed after a week and the spheres begin to show differentiation after two weeks. These organoids give rise to basal cells, goblet cells, and multiciliated cells. Cultured bronchospheres can be used as a great model to study human airway epithelium growth, repair, and differentiation. Their ability to differentiate in as little as 14 days could be useful for quickly making these organoids for experiments. The capability of culturing 3D airway organoids in 384-well plates can also be applied to a high-throughput system for drug screening.

Sachs et al. reported an alternative approach for long-term culturing of human airway organoids from bronchoalveolar resections or lavage fluids [53]. Isolated epithelial cells collected either from solid lung tissues or broncho-alveolar lavage fluid are embedded in basement membrane extract (BME). A 3D airway organoid with a polarized, pseudostratified airway epithelium containing basal, secretory, and multi-ciliated cells are formed within several days. This relatively simple protocol of generating airway organoids from a small amount of routinely obtained patient samples (lavages, resections) provides a great model for drug screening and personalized treatment of lung diseases [53]. These organoids were stable for up to several months and most retained the diseases, mutations, and tumours that the patients had. This model showed that personalized medicine for lung disease could be made a possibility with lung organoids.

Another adult stem cell-derived human lung organoid model is presented by Tindle et al. in which the generated lung organoids contain both proximal and distal airway epithelial structures [54]. Deep lung biopsy samples from patients are used to generate a single cell suspension followed by 3D lung organoid formation in Matrigel. An advantage of this human lung organoid model is that it recapitulates the proximal and distal airways, including all 6 major lung epithelial cells: AT1, AT2, basal cells, goblet cells, ciliated cells, and club cells. Besides culturing and maintaining in 3D culture, these lung organoids can also be dissociated and cultured as a 2D monolayer for viral infection studies. The 2D monolayer favoured differentiation from AT2 to AT1 cells, making this a great model to studying this process or for studying AT1 cells.

Recent advancements made by Salahudeen et al. have described a method for the development of long-term culture of human distal lung airway and alveolar organoids

[55]. They developed a feeder-free, chemically defined strategy to culture two types of human lung organoids derived clonally from single adult human AT2 cells or KRT5+ basal cells. The generated alveolar organoids are composed of homogenous AT2 cells capable of differentiating into AT1 cells while airway organoids contain two molecularly distinct distal airway basal cell subpopulations. Basal 1 cell population was characterized with proliferation and developmental programming. Basal 2 cell population was found to be enriched for structural, cytoskeletal, and calcium-binding protein genes. Both types of organoids were found to be stable for at least six months. The distal lung organoids were used to model COVID-19-associated pneumonia and since they were found to be useful in recapitulating disease, could be helpful for other lung diseases.

Youk et al. also described a long-term, feeder-free human 3D alveolar type 2 cell culture (h3AC) model derived from primary human lung tissue, which has a great potential in investigating the pathogenesis of SARS-CoV-2 and modelling other respiratory diseases [56]. After dissociating human AT2 (hAT2) cells obtained from distal parenchymal regions of healthy donor lungs and isolating through FACS using AT2 cell surface markers, sorted hAT2 single cells were embedded into Matrigel supplemented with growth factors that are essential in lung development to self-organize into an alveolar-like 3D structure. It was found that the differentiation from AT2 to AT1 cells was favoured in 2D culture. After six months, this 3D culture maintained normal karyotypes, but eight-month cultures lost some of their ability to form colonies as well as important markers such as the expression of pro-surfactant protein C (pro-SFTPC). Over several passages, h3ACs could still maintain functional mature hAT2 cells and were capable of AT1 cell differentiation when placed into 2D culture. Established h3ACs show a substantial SARS-CoV-2 infection with remarkable cellular and transcriptional changes post-infection compared to human 3D bronchial cultures generated previously by Sachs et al. and other 2D cell lines models [53, 57]. This 3D hAT2 cell culture serves as a great platform for viral infection studies to help better understand virus-host interaction and subsequent immune response in alveolar stem cells.

Lung Organoids Generated from Human Pluripotent Stem Cells (hPSCs)

Human lung organoids can also be generated from human pluripotent stem cells (hPSCs), including embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs). Generation of lung organoids from hPSCs usually requires a series of differentiation following developmental steps. In general, hPSCs are first specified to definitive endoderm (DE), followed by patterning to anterior foregut endoderm (AFE) and finally induced to lung lineage specification and maturation. These hPSC-derived lung organoids have great potential in providing models for studying human lung development, drug screening, and personalized medicine of various pulmonary diseases.

The first successful generation of AFE from hPSCs was achieved by Green et al. [58]. DE is induced from hPSCs with a high concentration of Activin A for four days and is confirmed with expression of DE markers CXCR4, c-KIT, and EPCAM [59]. To obtain an enriched culture of AFE cells after DE induction, Green et al. blocked

bone morphogenetic protein (BMP) and transforming growth factor beta (TGFβ) signalling using NOGGIN and SB-431542 (SB), respectively. This group found that the removal of Activin A allowed for an increase in SOX2 and CDX2 expression, markers for anterior and posterior endoderm, respectively. They attempted to further differentiate the AFE cells by replacing NOGGIN/SB-431542 with WNT family member 3a (WNT3a), keratinocyte growth factor (KGF), fibroblast growth factor 10 (FGF10), BMP4, and epidermal growth factor (EGF). This led to the increase of markers P63, NKX2.1, NKX2.5, PAX1, and a decrease in SOX2. The addition of retinoic acid (RA) increased lung fate markers GATA6, FOXJ1, NKX2.1, and FOXP2. This study was important for future researchers to continue the process of developing lung organoids, as a majority of groups furthering the development of lung organoids used these growth factors and markers to advance the maturation of lung organoids. Huang et al. followed shortly by differentiating AFE cells into lung and airway progenitors and was able to achieve a higher progenitor yield. The differentiated lung and airway cells could further differentiate into basal, goblet, club, ciliated, AT1, and AT2 cells in vivo and in vitro [60, 61]. They used the protocols discussed by Green et al. to generate DE but used dorsomorphin (DSM) instead of NOGGIN and added IWP2, a WNT inhibitor. Huang et al. optimized the protocol inducing lung progenitors from AFE by exposing the cells to the same growth factors and the addition of CHIR99021 (CHIR), a glycogen synthase kinase (GSK) inhibitor. This increased the amount of NKX2.1+ FOXA2+ cells by almost 20% [60]. The cells were plated on fibronectin-coated plates and cultured with the previously mentioned growth factors to induce the lung progenitors to mature. The concentration of RA was changed along with the culturing time which increased FOXA2+ NKX2.1+ cells from less than 40% to over 80%. This continuation of Green et al.'s research significantly improved the maturation of cells into AFE, allowing for the differentiation into lung and airway cells.

Dye et al. reported a protocol to generate 3D lung organoids with a proximal airway-like structure along with distal alveolar-like epithelial structure composed of the basal, ciliated, club and alveolar cells [62]. Cells were treated with Activin A, followed by NOGGIN/SB, and then the addition of CHIR, FGF4, SB, and NOGGIN to generate AFE. These AFE cells were then stimulated with a hedgehog (HH) while inhibiting FGF using Smoothened agonist (SAG) and SU5402 (SU), respectively [62]. This resulted in spheroids that were NKX2.1+ FOXA2+ which were placed in Matrigel. These spheres were added to decellularized human lung matrices, allowing for multiciliated structures. They called these human lung organoids (HLOs) and HLOs to contain a small population of AT1 and AT2 cells and alveolar progenitor cells. Human lung organoids resemble human fetal lungs based on their global transcriptional profiles, making them a great model system for human lung development [62].

Chen et al. described a different strategy to generate lung bud organoids (LBOs) that can form airway and early alveolar structures to recapitulate human fetal lung development [63]. hPSCs were differentiated into DE and AFE in the same manner as shown by their previous work [60]. When adherent AFE cells were induced to ventral AFE, cell clumps spontaneously formed LBOs and expanded further when

treated with FGF10, FGF7, BMP4, RA, and CHIR. These LBOs were cultured in suspension until day 20–25 followed by embedded in Matrigel for further generation of branching morphogenesis and maturation to lung and airway epithelial cells. LBOs can be cultured for an extended period of time, with maintenance capabilities in culture of more than 6 months. Both structural and transcriptomic data indicate that day 40 LBOs had reached the late second trimester of human gestation [64, 65]. LBOs were also transplanted under the kidney capsule of immunodeficient mice to determine if they could recapitulate lung development in vivo. This led to exhibited significant growth of airway structures undergoing branching morphogenesis showing proximodistal specification with evidence of early alveolar structures demonstrated by AT1 and AT2 cell markers [63].

McCauley et al. established a protocol to generate functional and expandable airway epithelial organoids [66]. Similar to other lung organoid generation approaches, hPSCs are first differentiated to DE, AFE, and then induced specifically to NKX2.1+ lung epithelial progenitors. These lung progenitors are then purified through FACS using cell surface markers CD47 and CD26 to sort out a high expression of CD47 and low or no expression of CD26. This is because cells that are $CD47^{high}CD26^{low}$ have a high-level NKX2.1+ cells. The isolated progenitors can then be re-plated in Matrigel with FGF2, FGF10, corticosteroids, and cyclic-AMP to form 3D airway epithelial organoids. These organoids express secretory lineage markers and airway basal cell markers. In addition, Kotton's group also developed a strategy to generate isolated hPSC-derived alveolar epithelial type 2 cells (iAT2s), which can form 3D alveolospheres [67]. Using surfactant protein C (SFTPC) as a specific AT2 cell marker and NKX2.1 as a lung progenitor marker to establish SFTPC/NKX2.1 multifluorescent reporter hPSC lines, Jacob et al. purified SFTPC+ iAT2s differentiated from NKX2.1+ progenitors. This confirmed that SFTPC+ cells derive from NKX2.1+ cells. iAT2s were shown able to self-renew and proliferate to form 3D alveolospheres with mature AT2 functions, including the formation of lamellar bodies and the secretion of surfactants. This was in contrast to primary AT2 cells, which required mesenchymal feeders in order to form spheres. This was an important discovery because primary AT2 cells are difficult to maintain undifferentiated [68], allowing for alternative methods for studying AT2 cells.

Miller et al. designed a protocol to generate lung organoids from feeder-free hPSCs, specifically hESC cell lines H1 and H9 along with hiPSC cell lines UM63-1 and UM77-1 [51]. This protocol is capable of forming bud tip organoids as early as day 22 and HLOs after 50 days. Briefly, to generate both organoids, hPSCs are directed to the endoderm followed by foregut spheroids. These spheroids float into the media, are placed into Matrigel, and cultured for two more weeks to generate bud tip progenitor organoids. If the bud tip progenitor organoids are not passaged and allowed to continue growing in Matrigel, they become budded structures. Those that are passaged form bud tip progenitors. The budded structures can become bud tip progenitors if passaged as well. The bud tip progenitor organoids are useful for research involving undifferentiated cells, as they are similar to the human fetal lung progenitors found on the branching buds [51]. Human lung organoids, found to be similar to the human fetal lung, contained matured alveolar cell types such as AT1 and

AT2 cells as well as mesenchymal cells. After being in culture for over 65 days, they will also be positive for basal stem cell marker P63+. Bud tip progenitor organoids are positive for SOX2 and NKX2.1 and, if passaged, will be SOX2+ SOX9+ [51]. Budded structures of these progenitor organoids undergo bifurcation and are positive for club cells, goblet cells, and pro-surfactant protein C.

Recently, a different protocol was described by Carvalho et al. to direct hPSC differentiation into mature lung and airway epithelial cells [69, 70]. By generating NKX2-1+ lung progenitors first in 2D cultures followed by embedding cells in collagen I without inhibiting glycogen synthase kinase 3, they could generate a more mature multilineage of alveolar and airway cells including AT1 and AT2 cells as well as basal, ciliated, club and neuroendocrine cells [69, 70]. Notably, KRT14+ NGFR+ (a mature basal cell marker) basal cells are formed following this protocol, which could also be easily isolated and expanded for subsequent basal cell culture. This protocol was built off of their previous work [61]. They used collagen I in place of Matrigel due to the fact that it could allow for a broader range of lung lineages but found that it produced similar lineages to protocols that use Matrigel. They found that NOTCH signalling induced a distal cell fate whereas WNT signalling induced a proximal cell fate. When cells that were placed in collagen I did not have GSK3 in culture, the cells matured into AT1 and AT2 cells. Better understanding signalling pathways involved in lung maturation has been crucial to better understanding lung development and the specification of different lung areas for experimentation.

Comparison Between hPSC- and Primary Stem Cell-Derived Lung Organoid Models

Several protocols have been developed using either primary stem cells, including adult stem cells (ASCs) and fetal stem cells or hPSCs to generate 3D lung organoids that can mimic the morphological and functional features of the human lung in vitro. Both organoid generation systems have their own advantages and limitations.

Human primary stem cell-derived lung organoids are usually generated from biopsies directly isolated from healthy or diseased patients' lungs. These organoids are often limited by the shortage of primary tissues and difficulty in accessing them. Heterogeneity among donors and unclear information of prior culture/preservation conditions of primary tissue present as limiting factors in primary stem cell-derived organoids. An advantage of using lung organoids from primary human tissues is that they are valuable for rare diseases such as CF to allow for drug modelling and better understand how the CFTR mutation will affect a particular patient [71]. On the other hand, hPSC-derived organoids can be used continuously to generate different models once a protocol is established. There is no concern as to where to obtain samples since they are derived from cells that can be purchased commercially. These organoids can be useful for a wide variety of diseases and have been found to recapitulate diseases successfully.

Another difference between primary stem cell- and hPSC-derived organoids is that primary stem cell-derived organoids are preserved to differentiate towards a certain lineage, such as proximal/airway or distal/alveolar lineage, and cannot trans-differentiate into others by just changing the culture environment. In contrast, the

lineage determination of hPSC-derived organoids largely depends on the external manipulation of signalling pathways and components in the culture medium. Therefore, hPSC-derived lung organoids usually contain a mixture of proximal and distal cells with a greater cellular heterogeneity, while ASC-derived lung organoids are limited to certain lineages depending on the primary tissue used. Nevertheless, the flexibility and uncertainty in hPSC differentiation can raise issues of certain lineage specification and inclusion of unwanted cell types in the culture. Both methods are complementary and can be used depending on the end goal of the experiments. ASC-derived lung organoids are useful to study specific cell types impacted by a particular lung disease. hPSC-derived organoids offer a more versatile approach to studying a wide range of cells and how a wide variety of diseases impacts the proximal and distal cell types.

An important advantage regarding the use of hPSCs is their ability to be easily genetically modified: isogenic cell lines with specific mutations can be generated using CRISPR-Cas9 and these genetically modified hPSC-derived organoids can then be used to model multiple respiratory diseases such as CF [72] and idiopathic pulmonary fibrosis (IPF) [65]. CF is known to be caused by mutations in the CFTR [73]. A study using a CRISPR-mediated gene editing approach successfully targeted and corrected the endogenous CFTR locus in CF iPSCs [72]. The gene-corrected iPSCs could later be differentiated into mature airway epithelial cells with normal CFTR expression and function [72]. Using this method of genetically modifying hPSC-derived lung organoids, more studies of lung diseases can be done. An example of a potential disease to study could be Hermansky-Pudlak syndrome (HPS), a rare autosomal disorder. patients with mutations in HPS genes have been found to develop HPS, specifically those with a mutation in the gene HPS1 [74]. They found that the mutation in HPS1 showed a high incidence of developing pulmonary fibrosis [74]. Mutations in some HPS associated genes can cause HPS-associated interstitial pneumonia (HPSIP), which resembles IPF [74]. HPS-associated mutations could be introduced into hPSC-derived 3D lung organoids using CRISPR-Cas9 to study the potential pathogenesis of IPF caused by HPS mutations [65]. It would be more difficult to obtain primary tissue from patients with HPS, but if possible, the research could also be performed on primary tissue to better understand how this mutation impacts the lungs.

Currently, the maturation of hPSC-derived lung organoids to adult stages remains a challenge. Most organoids show a transcriptome profile similar to embryonic developmental stages. Adult airway-like structures can only be generated after in vivo xenotransplantation, and most in vitro hPSC-derived lung organoids cannot mature beyond the second trimester of human gestation. While hPSC-derived lung organoids are closer to the human fetal lung, primary stem cell-derived lung organoids recapitulate features of adult lungs better in terms of maturity. A combination of primary stem cell- and hPSC-derived organoid systems would provide a more comprehensive understanding in human lung development and regeneration. Both primary tissue and hPSC-derived lung organoids have their advantages and disadvantages, but ultimately both are crucial to the advancement of lung research.

Mouse and Human Lung Difference

Animal models, especially mouse models, have greatly improved our understanding in lung development and disease. Mouse genetic gain- and loss-of-function studies enable us to learn more about lung development and signalling pathways controlling morphogenesis [40]. Mice lung injury models can reproduce some key features in complex human pulmonary diseases such as ALI and pulmonary fibrosis [75]. Although mice have been widely used for studying human lung development, function, and various respiratory diseases, it is worth noting that there are significant interspecies differences between mouse and human lungs. Given the significant differences, mouse models cannot fully recapitulate human lung physiology nor be applied to human lung development and disease studies. Some promising findings from mouse models fail to translate into effective therapeutic targets in subsequent human studies [76].

Cellular Composition

In both mouse and human lungs, the trachea and proximal conducting airways are lined by pseudostratified columnar epithelium, and the peripheral conducting airways are lined by cuboidal epithelium. Despite the similar structure in airway epithelia, the relative proportions of different types of cells along this proximal-distal axis of the airway vary between human and mouse lungs. In addition, this complex pseudostratified epithelium structure extends to terminal bronchioles in humans, whereas this structure is limited to the trachea and more proximal airways in mice. In human lungs, the more proximal intrapulmonary airways are lined by tall, pseudostratified, columnar epithelium composed of basal, ciliated, club, serous, mucus, intermediate, and neuroendocrine cells. These airways also exhibit abundant submucosal glands. In mouse lungs, however, the more proximal intrapulmonary conducting airways are lined by low columnar epithelium composed mainly of ciliated and club cells with some clusters of neuroendocrine cells. No basal cells and only a few mucus cells are found in the mouse airways [77]. Basal cells marked by the expression of transcription factor TP63 are only found in the mouse trachea, while the distribution of these cells extends to the bronchi in human lungs [52].

Architectural Organization

Both human and mouse lungs consist of multiple lobes but vary in numbers and organization. Mouse lungs have one lobe on the left and four on the right, while human lungs have two lobes on the left and three on the right [78]. For each lobe in the human lung, extensive interlobular and segmental connective tissues are separated into individual lobules or segments, while no such subunits exist in the mouse lung [79]. The alveoli and blood-air barrier are smaller and thinner in mouse lungs compared to that of humans [80].

Molecular Characteristics

Differences in the expression of several marker genes have been observed during human and mouse lung development. SOX2 and SOX9 are two essential transcription factors in lung development. During the pseudoglandular stage in developing mouse lungs, there is a clear separation between Sox9+ tip and Sox2+ stalk cells. These cells are formed and regulated through multiple signalling mechanisms [81]. This tip-stalk demarcation can also be seen in human fetal lungs; however, some levels of SOX2 are also found to be expressed with SOX9 in human embryonic lung distal tip epithelium. This co-expression pattern is never found in mice while the SOX2+ SOX9+ progenitor population persists during the pseudoglandular stage up to 16 weeks' gestation during human lung development [82]. The maintenance of this SOX2+ SOX9+ progenitor population is also proposed for proper branching morphogenesis in the human lung [37].

Lung Organoids: Potential Applications in Lung Repair and Regeneration

Lung disease is a major cause of morbidity and mortality worldwide. For many patients with end-stage lung diseases, lung transplantation remains the only available therapy. However, the number of patients listed for lung transplantation surpasses the number of suitable organ donors. Understanding the cellular and molecular mechanisms driving lung regeneration and repair is crucial for the development of novel therapeutic approaches, with the ultimate goal to repair the damaged lung in situ or regenerate the damaged lung for transplantation. The lung is a highly quiescent organ, previously thought to have a relatively limited reparative and regenerative capacity [83]. It is now known that following injury, the lung has a robust ability to repair and regenerate through distinct cell types. The ability to replace defective cells with cells that can engraft, integrate, and restore lung functions could be the potential cure of a number of lung diseases (Fig. 2.3).

1. Proximal airway repair and regeneration

The proximal airways serve as the first line of defence in the respiratory system as they are exposed to frequent insults from the environment. They consist of the trachea: a pair of primary bronchi and many bronchioles of various sizes generated through the branching morphogenesis process [84]. The proximal airways are lined by a pseudostratified columnar epithelium consisting primarily of three types of epithelial cells, (basal cells, club cells and ciliated cells) that play crucial roles in tissue repair, mucociliary clearance (MCC), and host defence. They also contain a small number of neuroendocrine cells, goblet cells, ionocytes, and tuft cells [12, 85–88]. The primary method of defense against the external environment is mucociliary clearance which requires the cells involved to be working properly [89]. If MCC in the lungs is nonfunctional or damaged, the lungs become vulnerable to other

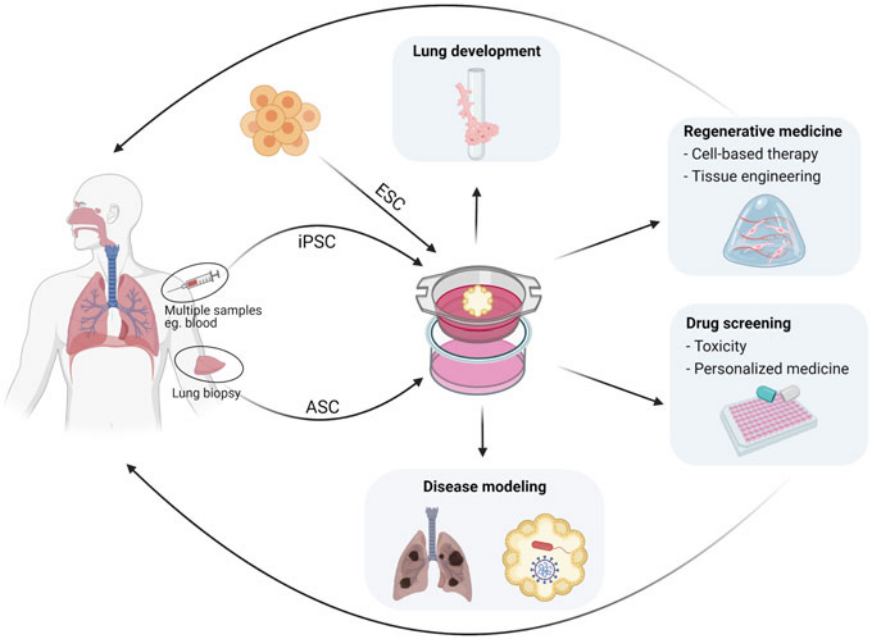

Fig. 2.3 Lung organoids: potential applications. As mentioned earlier, lung organoids can be established directly from patients via fresh biopsies and resected lung tissues, blood samples, and skin samples. Lung biopsies and resections contain adult stem cells (ASCs), whereas blood and skin samples contain induced pluripotent stem cells (iPSCs), which can be reprogrammed into the desired cell type. Regardless of the type of sample obtained, all can be differentiated towards the desired lineage. Lung organoids can also be derived from embryonic stem cells (ESCs). Lung organoids provide unique opportunities for (1) basic research: including studies of lung developmental processes, responses to external stimuli and stress signals, cell-to-cell interactions and mechanisms of stem cell homeostasis; (2) drug screening: in which patient-derived organoids can be used to predict how patients will respond to drugs; (3) disease modelling: to understand the mechanisms of lung diseases such as infectious diseases, inheritable genetic disorders, and cancer; (4) regenerative medicine: their capacity to engraft and survive *in vivo*; their ability to self-organize to complex structures resembling mini-organs *ex vivo*; and their potential to generate bioengineered tissue makes them optimally suited for regenerative medicine.

infections, making treatment even more challenging. An example of the MCC in the lungs not functioning properly since birth is in primary ciliary dyskinesia (PCD), a genetic disorder that causes motile cilia dysfunction crucial for MCC [89, 90]. These patients are more likely to get respiratory infections and are at risk for severe lung damage to the point of needing a lung transplant [91]. Examples of illnesses that cause damage to the lungs are the previous outbreaks of severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) that have shown to have long-term consequences on patients [92–94]. This raises concerns for the current SARS-CoV-2 pandemic since there is no way to obtain evidence for long-term consequences for patients at the moment. This concern supports the idea of

utilizing all possible ways of studying lung disease and repair; lung organoids can be pivotal in helping the lung field answer important questions.

The mouse trachea closely resembles the structure of human proximal airways, providing a valuable tool to study airway regeneration. Pioneering studies from Rock et al. [52], have shown that basal cells function as proximal airway epithelial stem cells. During physiological cell turnover or after injury, basal cells can self-renew and differentiate into various airway epithelial cell types for maintaining the epithelial integrity of proximal airways [52, 95]. Basal cells in the proximal airways are the major stem cell population that self-renew and, when necessary, give rise to multiple cell types such as secretory, goblet, and multi-ciliated cells [52, 95–97]. However, striking differences are also found between mouse and human lungs, as we discussed in the previous section, with the most relevant being the presence of basal cells [98]. In mice, basal cells reside in the main trachea, whereas, in humans, this population extends for several airway generations. Unlike human lungs, the intrapulmonary airways of mouse lungs are not pseudostratified and lack basal cells, highlighting the interspecies difference [76]. Organoids derived from human cells could provide an in vitro model for regenerating the mucociliary epithelium from basal cells. Using human lung organoids would provide a better model and give researchers more confidence to translate their results to patient care.

To model proximal airway functions, lung organoids have been derived from human and mouse basal cells. Depending on their origins, basal organoids derived from tracheal cells are known as tracheosphere while those from from large airway cells are called brochospheres in humans [52, 99–101]. These organoids have been used to test regenerative mechanisms proposed from in vivo studies. They can also be used to screen for drugs, small molecules, and molecular pathways participating in pulmonary cellular plasticity and lineage outcomes, and regulating crucial epithelial cell functions. For instance, Gao et al., used human basal cells derived organoids to identify a central role for the transcription factor grainyhead-like 2 (GRHL2) in coordinating barrier function and differentiation. Using CRISPR/Cas9 genome editing, they further revealed the transcription factor ZNF750 as a new component of the ciliogenesis pathway in the human lung [102]. If more lung organoids are used to study human disease, they will be the main method to fill the gaps of knowledge that hold research back from finding cures to lung diseases.

While studies have shown that basal cells are essential in repairing the damaged airway epithelium, other epithelial cells also participate in tissue repair as facultative stem/progenitor cells. Studies in mice have shown that club cells that reside throughout the airway epithelium are facultative progenitor cells [103]. Studies have shown that club cells can directly differentiate into mucus-secreting goblet cells by IL13 stimulation in both mice and humans, especially in more proximal lung regions [104]. Another example is PNECs which are neurosensory cells that spread sparsely throughout the bronchial epithelium and studies have shown that PNECs can self-renew and differentiate into club cells and ciliated cells following lung injury [105, 106]. Lung organoid technology can be applied to investigate the functions of these cells. For instance, using a 3D co-culture organoid system, Lee et al., demonstrated that Lgr5 and Lgr6 are markers of mesenchymal cells in the adult lung. Moreover,

these cells play important roles in direct airway differentiation of Scgb1a1+ progenitors and alveolar regeneration [107]. Organoid culture provides a model system for studying different airway epithelial stem/progenitor cells during repair, testing the effect of individual cytokines and growth factors on the proliferation and differentiation of secretory cells under pathological conditions, and identifying subpopulations of cells with enhanced regenerative potential.

2. Alveolar repair and regeneration

The alveolar epithelium of the lung is composed of two distinct epithelial cell types. AT1 cells cover 95% of the surface area of the alveoli and perform the function of gas exchange [108]. AT2 cells are characterized by the production of pulmonary surfactant proteins, which are essential for reducing the surface tension of the alveolar surface area to prevent the lungs from collapsing upon every breath [23, 108–110]. The alveolar compartment remains largely quiescent in the uninjured lung, and most cells within this niche exhibit a relatively slow turnover [88]. After lung injury, multiple alveolar cell types are able to proliferate and, when the repair is effective, alveolar structure and function are both restored [88]. Although the function of AT2 cells involves repair, this may not be enough to treat certain lung diseases where the cells no longer perform their standard roles. This is why research is still being done to unlock how these cells fulfill their many duties as the progenitors of the lung and recent studies have determined that this mechanism may be due to verying cell populations.

AT2 cells are the alveolar epithelial stem cells: they can react to injury involving both activations of self-renewal and differentiation into more mature cell lineages [109]. AT2 cells can form alveolospheres and differentiate into organoid structures that contain both AT2 and AT1 cells [17]. Within the population of AT2 cells, there are subpopulations that play certain roles both in the human lung and organoids. A subset of AT2 cells that express the transcriptional target of Wnt signalling, Axin2, were identified by Zacharias et al. in the human lung and were found to be responsible for generating the majority of AT2 cell growth in human alveolar organoids [111]. Another group identified a population of adult distal lung epithelial progenitor cells with low Wnt/β-catenin activity with strong organoid-forming capacity, suggesting their role in the alveolar epithelial repair [112]. A recent study identified the damage-associated transient progenitors (DATPs) via lung organoid models [113]. The DATPs are distinct AT2-lineage populations that are required for AT2 cells to differentiate to mature AT1 cells [113]. Alveolar organoids have also been used to study trophic interactions between different cell populations in the distal airways. For instance, alveolar organoids have been recently used to provide functional evidence that multiple signalling pathways originate in $Pdgfra^{+}$ lipofibroblasts to influence AT2 cell self-renewal and differentiation into AT1 cells through mediating multiple signalling, including BMP, FGF, and WNT signaling [114−116]. These discoveries are pivotal to better understanding how AT2 cells perform their endogenous functions. Once fully understood, these subpopulation roles could be manipulated in such a manner that could aid in repair and regeneration.

The contribution of AT1 cells to alveolar epithelial repair has not been studied extensively. A small subgroup of Hopx + AT1 cells can dedifferentiate into AT2 cells and thus participate in alveolar repair [117, 118]. Further in-depth characterization of these AT1 cells is needed to better understand the regulatory mechanisms guiding AT1 to AT2 transdifferentiation. Lung organoids can provide a useful model for identifying the cell types to increase insights into alveolar epithelial stem/progenitor cells during a repair. It is known now that cell populations such as immune cells are activated or recruited to the alveolar niche following lung injury. More complex lung organoid cultures incorporating immune cells will allow us to study the contribution of these niche cells that drive alveolar repair.

3. Recapitulating lung damage, repair, and fibrosis with lung organoids

While lung organoids are still in the early stage of development compared to animal models or conventional cell lines, recent studies using lung organoid models have largely advanced our understanding of the underlying pathogenesis of distinct chronic lung diseases [119].

4. Idiopathic pulmonary fibrosis

IPF is the most common and lethal form among interstitial lung diseases (ILDs) [120]. IPF is characterized by progressive fibrotic scarring in the lung tissue surrounding the air sacs, which ultimately leads to dyspnea. The etiology and pathogenesis of this disease are unclear [121, 122] and existing drugs can only slow disease progression [123, 124]. Lung transplantation is an option for IPF patients and has been found to extend their life but lung donors are limited, leading to extensive wait times which can cost the life of the patient [125, 126]. These limitations have motivated researchers to establish in vivo models to help mimic IPF in hopes of gaining insight to how to treat it. Models such as the bleomycin-induced mouse model and others have some gross similarities to human IPF but they fail to faithfully reproduce the pathophysiology of the disease [127]. Therefore, understanding the common pathways and pathogenetic mechanisms of lung fibrogenesis using representative models is critical for developing efficacious therapies [121].

Human pluripotent stem cells (hPSCs) have been shown to generate functional alveolar epithelial cells [67]. CRISPR genome editing has been used to introduce IPF-related genes in hPSC-derived lung organoid cultures which lead to the formation of abnormal cellular and morphological structures, including enhanced accumulation of mesenchymal cells and collagen, recapitulating important features of IPF [63, 65]. This provided a platform to identify pathogenic mechanisms of IPF that are likely clinically relevant in vitro. Using 3D pulmospheres from patients with IPF, Surolia et al. revealed the role of vimentin intermediate filaments in restricting the invasiveness of IPF fibroblasts [128]. The development of 3D organoid models can be adopted to model some forms of lung fibrosis, human distal lung structures, functions, and cell and matrix interactions opening the possibilities for high-throughput in vitro drug efficacy and toxicity screening assays.

5. Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is a leading cause of morbidity and mortality worldwide. The pathogenesis of COPD has been linked to cigarette smoking and, more generally to environmental exposures, i.e. air pollution and toxicants. A genetic component, autoimmunity, and accelerated cell senescence are partly involved in the pathogenesis of COPD. COPD is a complex disease that can present with emphysema, chronic bronchitis, or both. In particular, the presence of mucus plugging associated with goblet cell metaplasia (GCM) contributes to cough, sputum production, and airway obstruction [129]. Most of the animal models employed for the study of COPD develop emphysema but not bronchitis and the knowledge we have on the mechanisms involved specifically in the origin of the overproduction of mucus is still limited.

Despite advances in the field, there is still much to be learned about cellular and molecular mediators uniquely involved in the onset of GCM in COPD, and the identity of stem/progenitor cells in the human lung and how deficient repair may contribute to COPD. Recent data suggests a significant amount of plasticity in the lung, and the source of cells contributing to the increased numbers of goblet cells in COPD is currently not clear. Lung organoids may be a useful model to explore these questions. For instance, bronchospheres have been used to show that NOTCH inhibition limits goblet cell metaplasia in vitro [101]. Using lung organoid cultures, a previous study showed that upregulated noncanonical WNT signalling, through increased WNT-5a and -5b contributes to emphysema by negatively regulating alveolar repair [130]. Using alveolar organoids, Jacob et al. demonstrated that temporal regulation of Wnt activity could promote maturation of iPSC-derived AT2 cells [67]. These studies provide evidence supporting the use of alveolar organoids to explore the regulation of Wnt signalling in alveolar epithelial progenitor cells of COPD patients and to discover new treatment strategies.

6. Lung infection

Viral infections in the distal lung have been implicated in the progression of pneumonia to ARDS [131]. Respiratory viruses, including SARS-CoV-2, target lung epithelial cells, including AT2 cells [132]. Influenza viruses target AT2 and AT1 cells after intratracheal infection in mouse models [133]. However, there are currently no reliable models that recapitulate the phenotypes of lung infections in vitro. Lung organoids derived from hPSCs offer remarkable models to study the impact of different viruses including measles virus, respiratory syncytial virus (RSV), and the human parainfluenza virus type 3 (HPIV3) infections [63, 64]. RSV mainly causes respiratory tract infection in infants, and no vaccine or effective drugs have been developed yet [134]. RSV-infected hPSC-derived lung organoid cells led to detachment and shedding of infected cells into the lung organoid lumens recapitulating important features of the RSV-infected human infant lung [63, 64]. HPIV3 is a prevalent cause of lower respiratory tract disease in children. Consistent with clinical observations, HPIV3-infected lung organoids showed no detectable change in tissue integrity nor shedding of infected cells into the lumen [64]. Importantly,

whole-genome sequencing of HPIV3 in the lung organoids was found to be identical to the virus isolated in the clinical settings, suggesting that no selective pressure exists on the virus in the organoids [64]. The virus behaved similarly in the organoid models as it normally does in human infant lungs, indicating that the organoids are an optimal model for this particular infection. Lung organoids may be the key to generating a vaccine or treatment for HPIV3 which would be a significant accomplishment in the lung research field. Other viruses have been studied with lung organoids and are found to be as successful as well.

Influenza virus infection represents a major threat to public health worldwide. Zhou et al., developed human ASC-derived airway organoids (AOs) which can morphologically and functionally simulate human airway epithelium. These organoid cultures provide a reliable model to predict the infectivity of different human influenza virus can potentially provide a universal platform for studying the biology and pathology of the human airway [135].

Finally, the current COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) targets lung epithelial cells, including AT2 cells [132, 136]. Lung organoids as well as alveolar and airway spheroids that strongly recapitulate the lung structure and cellular environment have been used to uncover the pathogenesis and screen efficient therapeutic agents for COVID-19 [55, 137–139]. These studies suggest lung organoids may serve as an authentic model for respiratory viral pathogenesis, providing a valuable tool to study host-pathogen interaction, infection in the lung, and mechanisms of how viruses spread in the lung.

7. Eyes to the future: Bioengineering of the whole lung

To date, the only available treatment for patients with a variety of end-stage lung disease remains lung transplantation. Approximately 5,000 lung transplants are performed annually worldwide, with equal numbers of patients on waiting lists [140]. However, there are not enough donor lungs to meet current or anticipated future needs. To meet the growing demand for transplants, a promising area of research is the regeneration of pulmonary tissue using ex vivo bioengineering methods.

Bioengineering of the whole lung ex vivo for transplantation is extremely complex due to the complexity of the lung. With the progress of regenerative medicine and stem cell biology, decellularized lungs have been used as native scaffolds for seeding cells to regenerate the lung. Early studies were mainly performed using mouse lungs and several methods were later developed to decellularize the lungs of rats, pigs, non-human primates, and humans to subsequently recellularize this scaffolds [141−146]. However, most of these strategies focus on epithelial cells without the endothelialization of decellularized lungs [147]. Thus, one of the major challenges in whole lung bioengineering remains the generation of functional pulmonary vasculature. Moreover, given the limit access of biological materials as scaffolds, an emerging idea is to create a hybrid lung scaffold that combines extracellular matrix (ECM) components with synthetic scaffolds. Finally, lung organoids combined with bioengineering could generate more complex and mature organoids that can be applied to developmental biology, personalized medicine and lung regeneration. We will discuss in more detail in the following section.

Lung Organoids and Personalized Repair

Lung diseases impose a great socioeconomic burden due to their high morbidity and mortality rate worldwide. With the limited effective treatments available in the last decade, developing novel therapies for pulmonary diseases is pressing. Lung organoids serve as one of the most promising modelling approaches to study patient-specific therapy and personalized medicine.

Bioengineered Lung

A growing number of tissue engineering techniques widens the potential of establishing more physiological relevant and functional human lungs ex vivo for transplantation. A common method to manufacture whole lung tissue ex vivo is to first derive decellularized 3D lung scaffolds from various species such as humans, pigs and rodents, and then reseed the scaffolds with patient-derived stem cells or primary lung progenitor cells [147]. The use of 3D scaffolds is beneficial as the native architecture of the lung is mostly retained following decellularization compared to *de novo* lung bioengineering [147]. A study done by Ghaedi et al. had shown the potential in human lung regeneration and lung transplantation via repopulated decellularized human and rat lungs with iPSC-derived epithelial progenitor cells [148]. The epithelial progenitor cells were found to perfuse in both airway and alveolar compartments of decellularized lung scaffolds to form a bioengineered ex vivo lung [148]. This lung regeneration approach could be applied in clinics by combining a native matrix scaffold with patient-derived cells to generate a personalized lung for lung transplantation therapies.

Wilkinson et al. developed another scaffold-based approach to generate self-assembled human lung organoids with the use of functionalized alginate beads under rotation in a bioreactor [149]. The engineered 3D lung organoids contained multiple cell types including pulmonary fibroblasts, small airway epithelial cells, and human umbilical vein endothelial cells [149]. By scaffolding mesenchymal cells into the interstitial space between hydrogel beads, this engineered lung organoid could recapitulate the anatomy of distal lung alveolar sacs [149]. They also showed the capability of this scalable iPSC-derived mesenchymal organoid culture approach to model IPF. Having the advantage of assembling organoids through different combinations of various types of cell-coated scaffold units, this organoid generation system could be personalized for patient-specific disease modelling and drug discovery [149].

3D-Printing Facilitated Precision Tissue Engineering

With the development of 3D printing technology, artificial organs with patient-specific spatial architecture have emerged as an attractive alternative for precision medicine. Customized implants with patient-specific size and shape can be accurately manufactured by 3D printing. These customized implants can perfectly fit the defect sites of patients to significantly reduce surgical operation time [150]. Grigoryan et al. have developed a bioinspired alveolar model using poly (ethylene glycol) diacrylate (PEGDA) and a stereolithographic printer, which contains regions

reminiscent of native alveolar air sacs and alveolar buds [151]. This distal lung model contains functional intravascular oxygen transport through measuring blood oxygenation entering and leaving the model [151]. Another research group generated a bio-3D-printed artificial trachea using a Regenova bio-3D printer to create a scaffold-free tubular tissue from multicellular spheroids [152]. After generating multicellular spheroids from the mixture of rat chondrocytes, endothelial cells, and mesenchymal stem cells, the aggregated spheroids are then assembled into trachea constructs in a needle array, and the artificial trachea can become mature in terms of chondrogenesis and vasculogenesis in a bioreactor. Taniguchi et al. showed that the scaffold-free artificial tracheas remain functional with sufficient mechanical strength after transplantation into an isogenic rat for several weeks [152].

Lung Cancer Organoid Models

Many researchers work hard on generating patient-derived 3D lung tumour models, including spheroids and organoids, to study personalized medicine. Li et al. have established 12 patient-derived organoid lines from lung adenocarcinoma (LADC), which recapitulate the 3D structure and retain the genetic mutations of parental tumors [153]. Li et al. established these LADC organoids through dissociating tumour cells isolated from LADC samples and cultured them in Matrigel. These patient-derived organoid lines can be used for tumour biomarker identification and high-throughput drug screening. Together, this LADC organoid biobank serves as a good model to generate personalized therapy.

There is still much more to be learned about lung organoids, but their potential applications in personalized medicine is in the near future. As of late, there is no research involving the direct use of lung organoids on humans, but multiple studies are using them to advance human health. Researchers have used lung organoids generated from fetus and infant tissues diagnosed with Bochdalek congenital diaphragmatic hernia (CDH) to better understand the disease [154]. This study showed that the lung organoids could model CHD ex vivo and provide better ways of studying human diseases without using deceased human tissues. Other researchers have been able to model lung cancer using tumor tissues ex vivo and study potential patient-specific drug responses by comparing the lung organoid responses to patient responses [155]. Hu et al. determined the potential of a 1-week on-chip drug sensitivity test to predict patient responses to anti-lung cancer drugs [155]. Although this study found that the patient responses correlated 100% with only 11 of the 21 organoid samples, it gives hope to the idea of personalized medicine using lung organoid models.

Although many challenges need to be addressed before realising precision medicine treatment for lung diseases, such as the lack of a vascular system in most lung organoid models, the future of engineering functional lung and transplanting engineered lungs to patients is very promising.

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Chapter 3
Lung Organoid: Innovative Technology for Respiratory Disease Modelling

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Abstract **Introduction**: The lungs are a complex organ with various cell types. Many factors can contribute to the damage of the lung epithelial cells, including exposure to air pollutants, cigarette smoke and pathogen. Thus, it is critical to developing suitable human disease models to understand tissue homeostasis mechanisms and pathological alterations in the airways. Three-dimensional organoids made from stem cells have emerged as a new method to model respiratory disorders in vitro. On-going studies show that lung organoids generated from adult lung stem cells and induced pluripotent stem cells provide an excellent platform for model lung diseases and drug screening that alleviates respiratory diseases. **Method**: MEDLINE/PubMed and Google databases were used for the selection of literature. The keywords used were lung organoids, respiratory disease models, stem/progenitor cells, induced pluripotent stem cells. **Results**: Lung organoids can be derived from human pluripotent stem cells and adult stem cells. The microenvironment for culture and starting cell types are essential in generating lung organoids. For creating 3D lung organoids, an extracellular matrix component such as matrigel, feeder cells, and lung fibroblast is essential to provide a cocktail of growth factors that significantly contribute to the development of lung organoid culture. **Conclusion**: From this perspective, we summarise the recent technology of cultivating lung organoids and their potential applications to study respiratory diseases, including idiopathic pulmonary fibrosis, cystic fibrosis, tuberculosis infection, and respiratory virus infection. We also discuss challenges that need to be overcome to apply lung organoids as respiratory disease models.

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Keywords Lung organoids · Respiratory disease models · Stem/progenitor cells · Induced pluripotent stem cells

Abbreviations

COPD	Chronic obstructive pulmonary disease
IPF	Idiopathic pulmonary fibrosis
ARDS	Acute respiratory distress syndrome
3D	Three-dimensional
CF	Cystic fibrosis
PSCs	Pluripotent stem cells
ASCs	Adult stem cell
iPSCs	Induced pluripotent stem cells
ESCs	Embryonic stem cells
AEC I	Alveolar type I cells
AEC II	Alveolar type II cells
ECM	Extracellular matrix
ALI	Air-liquid interface
MCACs	Multi-ciliated airway cells
MTB	Mycobacterium tuberculosis
CFTR	Cystic fibrosis transmembrane conductance regulator
SFTP	Surfactant protein gene mutations in the family

Background

The lung is a complex organ composed of a variety of cell types. Approximately 40 distinct cell types make up the human lung, which allows it to perform its fundamental function of efficient gas exchange [1]. These cells, which originate from all three germ layers, comprise epithelial cells, neuron cells, hormone-producing cells, interstitial connective cells, and blood cells. These cells combine to construct the intricate lung tissue architecture, ranging from blood vessels to branching alveolar structures [2].

In the lung, exposure to air pollutants, cigarette smoke, bacteria, viruses and many others may cause injury to the epithelial cells that lined the conducting airways and alveoli. Repetitive exposure to these insults may result in an inflammatory storm that contributes to the disease progression and respiratory failure. Chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), bronchial asthma, and respiratory infections like the current coronavirus COVID-19 pandemic and others are among the respiratory illnesses [3, 4] that cause severe damage to the lungs and leads to an increase in mortality rate. Therefore, establishing suitable models to study human diseases, particularly respiratory diseases, is critically important

in advancing our understanding of tissue homeostasis mechanisms and pathological changes in the airways. For decades, conventional two-dimensional monolayer cultures of immortalised pulmonary cell lines, primary cells, and numerous animal models have been employed to investigate lung malfunctions associated with respiratory diseases. These studies have been conducted to gain a better knowledge of normal lung development and disease-related lung dysfunction. However, they are restricted by the lack of total cellular variety in 2D culture formats and intrinsic variations in animal physiology in animal models. To overcome the constraints of monolayer cultures for modelling in vivo lung tissues, recent breakthroughs in stem cell biology have resulted in the in vitro establishment of miniature three-dimensional (3D) structures produced from stem cells known as 'organoid' structures.

Organoids are 3D tissue constructs generated from stem/progenitor cells that contain multiple differentiated cell types observed inside the original organ in an organised way. Their organ-like organisation is remarkably similar to that of native organs such as the prostate [5], liver [6], tongue [7], pancreas [8], stomach [9], and lung [10]. In respiratory diseases, lung organoids have significant potential as disease models that can develop new treatments for conditions such as asthma, cystic fibrosis (CF), and many others because organoids exhibit similar fundamental inherent patterning events as the original organ [11]. Furthermore, lung organoids can be generated from small patient tissues to create living biobanks that aid in personalised biomedical research. [12]. This article discusses the new technology of lung organoids and its use in modelling various respiratory illnesses, the problems and roadblocks that have been encountered and future possibilities for improving and expanding the technology uses.

Lung Injury and Repair

Lung injury can impair the vital physiological functions of the lung, which is commonly caused by several human respiratory disorders. Smoking reduces the airway epithelium's integrity and causes significant epithelial remodelling, linked to COPD and lung cancer [3]. Pathogen such as the recently discovered coronavirus COVID-19 causes an inflammatory storm that leads to the destruction of airway epithelium, resulting in acute respiratory distress syndrome (ARDS) [4]. Impaired epithelium following infection may diminish the airway's ability to combat infection by other pathogens, contributing to severe lung damage and prolonging the disease. Post-injury, the lung has a robust capability to repair and regenerate itself via various mechanisms, including the interaction of multiple cell types [13]. Thus, researching the response of damaged lung epithelial layers, such as cell-cell interaction during the repair process following injury and the regulatory mechanisms governing the different signalling pathways, may help us better understand respiratory diseases and their treatment.

Current models to investigate respiratory diseases or drug screening applications are limited in recapitulating the injured lung tissues. Adult lungs function as a result

of the synergistic interaction of diverse cell types in a 3D architecture. [14]. However, the majority of in vitro techniques currently use 2D monolayers. Although conventional monolayer cell cultures were commonly used in the past, their lack of tissue architecture and complexity rendered them incapable of reproducing in vivo cellular heterogeneity, structure, functions, and biological processes [15]. It has long been documented that removing cells from their natural environment and architecture and placing them in 2D environments causes them to lose their tissue-specific functions [16]. Furthermore, prior research has indicated the importance of architectural signals in the establishment and progression of cancer. As a result, a representative in vitro model systems must replicate the 3D architecture.

Animal models, such as mice, rats, rabbits, and large animals, offer intriguing opportunities for developing diverse lung injury models that bridge that gap between human and animal lungs. Researchers can use animal models to investigate the molecular mechanisms that control lung function in the healthy state and malfunction in the disease state, allowing for a comprehensive understanding of the genesis and pathophysiology of disease and the development of innovative treatments. Additionally, the animal model has been used to evaluate the safety and efficacy of therapies in the complex environment of a live animal's lung. There are currently numerous induced animal models of human respiratory illness accessible for research. These models mirror some, but not all, of the characteristics of respiratory disorders such as pneumonia, asthma, emphysema and pulmonary fibrosis [17]. Unfortunately, none of the animal models is entirely accurate representations of human lungs and diseases. Due to significant inter-species variances in the respiratory architecture system and genetics, all animal models have restrictions in replicating intricate clinical conditions. Mice, for example, lack a cough reflex, and their distal airway structure and cellular composition are distinct from those of humans due to the absence of cytokeratin 5+ basal cells, which comprise a population of pulmonary stem cells within the pseudostratified epithelium of terminal respiratory bronchioles [13]. As a result, human and mouse cells engaged in the distal airway and alveoli repairs may have different origins. Another important illustration of this concept is the inability to generate CF lung disease in mice, where the absence of submucosal glands in the CF animal may prevent the development of CF lung disease in the CF mouse [18].

While animal models of human respiratory disorders have been extensively employed, their findings have not always translated into safe and effective treatments in people, impeding clinical translation. Inter-species differences, such as genetics, homeostatic physiology, respiratory tree architecture, and inter-species functional variances, are a significant roadblock to overcome when looking for acceptable respiratory illness models [3]. For financial reasons and a lack of understanding of mouse genetics, the reliance on murine models may be short-sighted, as mouse models often do not adequately reflect human conditions. As a result, establishing more relevant, comparable, and valuable models that bridge the "laboratory bench to the bedside" could be a more effective strategy to solve this issue, and lung organoids might be a good fit.

Lung Organoid as an Alternative Approach Over Animal Model to Study Lung Diseases

Organoids are three-dimensional self-organising multicellular constructions derived from stem cells that simulate the architecture and function of actual organs in vivo [19], which can be generated using pluripotent and adult stem cells, respectively. Organoids derived from adult stem cells take advantage of the tissue regeneration process initiated by these cells. In addition, organoids can be cultivated directly from the epithelium of several organs, whether healthy or diseased and can be evaluated in the same way that cell lines are. Experimental biology applications include modelling tissue physiology and disease, including malignant, hereditary, and infectious illnesses [20]. Lung organoids, like other organoids, can be created by a self-organisation process from stem cells or lung progenitor cells. When compared to standard cell culture, the culture method of lung organoids in vitro is distinctive.

The lung organoids, which can be classified as proximal or distal, mimic the lung developmental process and strongly resemble the 3D organisation of the lung (alveolars, airways, and lung buds) and lung functions in vitro [21]. These organoids can be classified into two categories according to the type of stem cell used [(pluripotent stem cells (PSCs) vs adult stem cells (ASCs)] and the growth factor regimen used. To begin, pluripotent stem cells (PSCs) encompass both induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) [21]. These cells represent embryonic development's early phases. The second type of ASC is lung-specific, including alveolar type II cells (AEC II) and club cells. These stem cells are responsible for maintaining tissues and organs throughout a person's life. Their activity is limited to alveolar type I (AEC I) cell formation. However, they all share a common goal: to generate a model system that replicates vital morphological and functional characteristics of the in vivo pulmonary epithelium.

Current Technologies in Generating Lung Organoid

Lung organoids are produced using a flexible and regulated process regarding the starting cell types and microenvironments for culture, like culture media and culture systems. The essential component in forming lung organoids is the initial cell types, which define their final use. The culture microenvironments are also crucial in the formation of lung organoids. For creating 3D lung organoids, matrigel, a complex extracellular matrix (ECM), is required to provide cells with a support structure (similar to that of an organ) in vivo and enhance cell proliferation and differentiation in the culture environment. Matrigel is a natural basement membrane originally isolated from Engelbreth–Holm–Swarm mouse sarcoma cells and utilised in early lung organoid culturing [22].

Cultivation microenvironments for the generation of lung organoids are varies depending on the research strategies. The establishment of a 3D co-culture system

with primary PDGFRα+ lung fibroblasts by Barkauskas and co-workers found that the trophic effect of fibroblasts resulted in the rapid formation of more and rounder alveolospheres [23]. Jacob and colleagues generated monolayered alveolospheres in 3D cultures without the support of stromal cells [24]. The omission of supporting cells in the culture system did not affect the ability of the iPSC-derived AT2 cells to proliferate and differentiate. Air–liquid interface (ALI) is another culture system closely connected to respiratory physiology, resembling the pseudostratified mucociliary epithelial structure of airways [25]. The "3D-ALI" method, which combines ALI with Matrigel, produces lung organoids with realistic structure and function. Multiciliated airway cells (MCACs) function better in lung organoids generated from hPSCs using the 3D-ALI method than MCACs in a 3D culture [26]. However, this ALI system confines the spatial structure of the trachea only.

Lung Organoids Derived from Human Pluripotent Stem Cells

Human pluripotent stem cells are cells that can differentiate into any cell in the body. They include human embryonic stem cells and induced pluripotent stem cells (iPSCs), somatic cells that have been reprogrammed to become pluripotent [27, 28]. The endodermal germ layer is essential for the formation of the lung. In order to differentiate organoid lung tissues in vitro, pluripotent stem cells must be directed into the endodermal lineage by activating TGF-beta signalling using Activin A, which replicates the Nodal signalling that is necessary for mesendoderm determination in the embryo [29]. When WNT3A and FGF4 are added to a DE monolayer in vitro, cells take on a hindgut destiny and form spheroids separate from the adherent monolayer and float above it. These spheroids can be placed in an extracellular matrix-like matrigel, where they grow and develop in three dimensions, giving rise to larger organ-like structures known as lung organoids. [30]. Concurrent stimulation of DE cultures with spheroid-inducing factors (WNT3A/FGF4) and foregut patterning factors (NOG/TGF-inhibitors) resulted in the formation of foregut spheroids, which then expanded into larger lung-organoid structures. The expansion of hPSC-derived lung organoids was driven by data from animal development and work showing how to culture primary lung tissue in vitro. [31]. The ability of hPSC-derived tissues to self-assemble in vitro is an astounding but underappreciated mechanism, especially considering that in vitro systems frequently lack growth factor gradients considered crucial for tissue organisation in the developing embryo.

The use of human ESC and iPSC lines to generate organoids avoids the scarcity of high-quality human basic components while also necessitating a thorough understanding of the mechanisms in germ layer formation and subsequent lineage specification to carry out guided differentiation. When opposed to ESCs, using iPSC lines involves an extra step, as somatic cells must first be transformed into iPSCs by expressing transcriptional factors such as OCT4, KLF4, SOX2, and MYC. Following

that, ESCs and iPSCs are subjected to germ layer and tissue-specific patterning factors, followed by embedding in matrigel to aid 3D architecture advancement and treatment with differentiation factors to create the desired organoids. TGF signalling is activated in ESCs and iPSCs to generate definitive endoderm, differentiating into the appropriate embryonic gut segment depending on cultural circumstances [32]. Human ESC-derived endoderm was supplemented with a Hedgehog pathway agonist to promote the progressive devotion towards foregut endoderm and, eventually, spherical epithelial organoids expressing both proximal and distal lung markers observed during branching morphogenesis in vivo. Unlike those produced from raw foetal tissue, organoid cultures did not show any branching [33–35].

Rossant and colleagues were the first to produce lung organoids from human iPS cells, and they included the use of CFTR-mutant iPS cells as a proof of concept for modelling cystic fibrosis (CF) [36]. Snoeck and co-workers later generated lung bud organoids from human iPS cells that mimic fetal lung development [37]. Because hPSC tissues generated through directed differentiation are immature, they can be used to study human diseases that are immature or premature. Premature babies' respiratory and digestive systems attain further investigation using human model systems such as hPSC-derived human lung and intestine organoids [38]. Pluripotent stem cells have therefore been successfully used to develop organoids from non-epithelial tissues. Because iPS-derived organoids do not generate the mature cell types observed in adult tissues, they may not accurately replicate adult disease presentations in many cases [11]. This is especially true in lung cancer, where it looks to be a waste of effort to generate iPS cells from the tumour rather than directly produce cancer organoids. Organoids produced from ASCs mimic the more advanced stages of the human lung and may be made from a single patient's bronchoalveolar lavage material [3].

Lung Organoids Derived from Adult Stem Cell

Organoids generated from the adult stem and progenitor cells, in contrast to iPSC, reliably preserve their in vivo regeneration activity in vitro, allowing for comprehensive pictures of tissue repair following damage. These organoids made from adult stem cells preserve their organ identity and are genetically stable throughout time [39]. Organ development, tissue homeostasis, and illnesses may all be studied because of the capacity to generate organoids from patient-derived healthy and sick tissue. When lung fibroblast cells overgrow, their capacity to sustain lung stem/progenitors is diminished. These findings show that supporting fibroblasts' secretory characteristics are essential for such effective organoid culture of endogenous lung stem/progenitor cells. Previous research has found that a culture protocol for lung stem/progenitor cells that includes matrigel, feeder cells, and lung fibroblast to provide a cocktail of growth factors that contribute significantly in lung organoid culture harvested from fibroblast cultures is less supportive for distal lung stem/progenitor cell organoid culture, most likely due to insufficient concentrations of essential growth factors.

[23]. When stromal cells are replaced with large amounts of FGF10 and hepatocyte growth factor, lung stem/progenitor cells form organoids with limited colony-forming capacity, implying that additional growth factors are required for alveolar organoid formation [39]. Randomly seeded mixed cell populations of human adult primary bronchial epithelial cells, lung fibroblasts, and lung microvascular endothelial cells generate airway organoids self-organised into discrete epithelial and endothelial structures stable up to 4 weeks culture, according to a previous study [11, 40]. This discovery showed that a cocktail of growth factors given by supporting cells is critical for the rapid and robust generation of lung organoids.

Organoids in Respiratory Diseases Modelling and Personalised Medicine

Lung organoids are a powerful new technique because they reliably replicate primary tumours, faithfully recapitulate treatment responses, and even help optimise therapeutic methods for each patient as near-physiological structures. Furthermore, healthy organoids can be used to evaluate medication toxicity, including hepatotoxicity, cardiotoxicity, and nephrotoxicity. Lung organoids have been widely used in various applications, including drug discovery and disease modelling, precision medicine, and regenerative medicine. This chapter focuses on organoids in the modelling of viral illnesses, hereditary disorders, and lung cancer.

Infectious Disease

Organoids generated from adult stem cells have been employed in several studies to investigate host-pathogen interactions [41]. Most pathogens enter organs through the lumen and touch the epithelium's apical surface, where differentiated cells dwell. One of the essential advantages of utilising organoids to investigate host-pathogen interactions is that they are made up of or developed into almost all of the various cell types seen in a given organ. They are thus preferable to immortalised cell lines that have previously been frequently used to simulate host-pathogen interactions because they closely resemble the in vivo environment [42]. For example, animal models used to study tuberculosis pathologies and drug screening have numerous limitations in Mycobacterium tuberculosis (MTB) infection. Since the animals are not natural hosts for MTB, they can only mimic the clinical symptoms, pathological abnormalities (granuloma formation and lung cavitation), and immunological indications of tuberculosis to a limited extent [41].

As a result, lung organoids are gaining popularity as a tool for studying the interaction of host-MTB in a dish. The spatial organisation of human lung organoids and

the variety of their cellular components provide a significant advantage. MTB infections of alveolar organoids enable the inclusion of early-stage MTB infection that is difficult to track in animal models while also overcoming species differences [43]. Human alveolar organoids can be used to evaluate the direct interactions between MTB and the lung epithelium in this condition by injecting MTB into produced organoids [44]. Additionally, immune cells, including macrophages, can be incorporated into the organoid architecture to imitate the complexity of the immune response in vivo.

With the organoid-bacteria co-cultures mentioned previously, adult stem cell-derived organoids have been utilised to model viral infections [45]. Virus infections in the distal lung have been associated with pneumonia's acute respiratory distress syndrome (ARDS) progression. Lung epithelial cells, particularly alveolar type II cells (ATII), are targeted by respiratory viruses, including the recent SARS-CoV-2 [44, 45]. In animal studies, influenza viruses specifically target AT2 and alveolar type I (AT1) cells subsequent intratracheal infection [46]. The absence of functional models that match in vivo physiology and pathology has hampered research into human respiratory infections. In vitro organoid cultures are unique model systems for studying disease aetiology and host-virus interactions. Han et al. [44] previously developed a lung organoid model that is tolerant to SARS-CoV-2 infection using human pluripotent stem cells (hPSC-Los) and has shown intense chemokine stimulation of SARS-CoV-2 infection, akin to what is observed in patients with COVID-19 [47].

According to another study, SARS-CoV-2 invaded and propagated in lung organoids derived from human embryonic stem cells (hESCs), both airway and alveolar organoids. They also conducted pharmacological screening, identifying SARS-CoV-2 entry inhibitors such as mycophenolic acid, imatinib and quinacrine dihydrochloride. When these medications were administered at physiologically acceptable levels, the scientists found that they significantly reduced SARS-CoV-2 infection in organoids [47]. They also discovered that suppressing SARS-CoV-2 propagation in lung organoids was successful using camostat, a nucleotide analogue prodrug similar to Remdesivir [48]. Thus, these findings suggest that human lung organoids could be used as disease models to study SARS-CoV-2 infection and are a valuable resource for drug development and screening to discover a COVID-19 treatment option.

Previous research revealed that the human parainfluenza virus 3 (HPIV3) infected AT2 cells in lung organoids produced from human pluripotent stem cells (hPSCs) [49]. In line with clinical evidence for HPIV3 infection, no alterations in tissue integrity or shedding of infected cells into the organoid's lumen were seen [49]. A histological examination of respiratory syncytial virus (RSV)-infected human lung organoids generated from hPSCs revealed considerable epithelial alterations that mimicked *in vivo* pathologies, such as apical protrusion of infected cells cytoskeletal rearrangement and formation of syncytia [37]. Palivizumab, an antibody that inhibits RSV entry into the airway, and other antiviral agents may also be assessed for their antiviral ability in alveolar organoids. Similar organoids have been produced to rapidly assess the human pathogenicity of new influenza viruses [50]. Thus,

such organoids can be used to investigate host-pathogen interactions in a range of lung infections and provide a pathophysiological model for pathogen infection and treatment discovery.

Genetic Disease

Organoids can be used as a one-of-a-kind platform for studying the biology of hereditary lung disorders. For example, organoids from cystic fibrosis patients have been successfully created to investigate possible treatment methods [11]. According to a compound screen using an organoid as a platform, two types of small-molecule compounds, including cystic fibrosis transmembrane conductance regulator (CFTR) correctors for improving cellular processing and CFTR potentiators for enhancing the performance of the CFTR protein's gating function, effectively rescued the CF phenotype [11]. It was also shown that CFTR function was restored in organoids by adding chemically modified mRNA to the CFTR gene. As a result, the newly developed culture system provides a unique method for genetic disease medication screening.

Lung organoids may aid in the understanding of the fibrotic lung disease associated with familial genetic defects [39, 51, 52]. The most common type of interstitial lung disease (ILD) is idiopathic pulmonary fibrosis (IPF). IPF is characterised by scarring of the lung alveoli, which, if left untreated, would result in alveolar stiffness and respiratory failure [51, 52]. Although the aetiology of this disease is unknown, genetic susceptibility is one of the risk factors for alveoli collapse. Surfactant protein gene mutations in the family (SFTP) [51, 52] and mutation in human reverse transcriptase (hTERT) and its RNA component (HTERC) of telomerase can be captured using lung organoid modelling of alveoli from the IPF patients [51]. The association of IPF pathogenesis and mutated genes of telomerase activity will suggest the role of type II alveolar cells to function. In 2019, Strikoudis et al. modelled pulmonary fibrosis using lung organoids derived from embryonic stem cells carrying Hermansky-Pudlak syndrome mutations (HPS) [51]. A recessive mutation in several genes associated with this condition results in aberrant lysosome-related organelle biosynthesis and trafficking [24].

Additionally, HPS-associated interstitial pneumonia presented clinically similarly to IPF [51]. The introduction of all these HPS mutations to the lung organoids promotes fibrotic changes, and these modifications indicated that interleukin-11 plays a critical function in the fibrotic process [51]. Using lung organoids as an alternate approach to recreate the chronic progressive damage found in humans with IPF, which animal models do not duplicate. To show genuine development in the IPF disease paradigm, the bleomycin-induced mice model requires continuous infusion and long periods [53, 54].

Some forms of surfactant disorders, like adult ARDS, desquamative interstitial pneumonitis (DIP), and diffuse lung disease, are caused by mutations in genes affecting surfactant homeostasis, notably SFTPA, SFTPB, SFTPC, ABCA3, and

CSF2RA (DLD) [55]. This condition results from the failure of the surface tension control system to keep lung volumes constant at the end of expiration [51, 55, 56]. The ability to control surface tension is vital to the type II alveoli cells inflating during inhalation and preventing lung collapse [51]. Current findings suggest that footprint-free CRISPR-based gene repair of iPSCs originating from patients with homozygous surfactant mutations (SFTPB) recovers surfactant processing in type II alveolar cells. The creation of structurally specialised lung organoids, such as alveolar organoids, can assist researchers in better understand the role of pulmonary surfactant failure in ILD patients' genetic mutations caused by type II alveoli cells [24, 39, 52]. Jacob et al. using epithelial alveolosphere in 3D cultures to model the defective gene in type II alveoli from the neonatal respiratory distress syndrome who are homozygous for the SFTPB mutation [39]. Taken together, the ability of alveolar organoids to simulate human alveolar disease in vitro may provide a valuable platform for further investigation of the impact of genetic and environmental insults on type II alveolar biology.

Cancer

Lung cancer is the leading cause of cancer death worldwide. Because other lower-risk conditions might conceal its symptoms, this pathology's high death rate is directly proportional to its late identification. Although many studies on this topic have expanded in recent years, the molecular pathways that cause this disease are still unknown. As a result, experimental models are critical for understanding disease development, progression, and response to therapy. Immortalised cell lines come with their own set of drawbacks. Explanted tumoral cells obtained during transthoracic needle biopsy could be a source of human lung tumour cells for primary culture. As a result, cancer organoids derived from human cancer tissues have been suggested as an alternative in vitro model that retains the features of the original tumours. These cancer organoids may serve as a model for selecting anticancer therapy and biobanking for individual patients.

Through precision medicine, the organoid platform enables us to investigate the interactions between tumour cells and the controlled surrounding ECM, thereby improving our understanding of the underlying processes of drug resistance [57]. We may potentially use the organoid culture technique to anticipate in vivo tumour responses to anticancer reagents and test the most effective medicines in customised cancer treatment [58]. It is critical to use approaches that allow for the examination of lung function in both normal and pathological stages while utilising the entire lung architecture to understand these processes thoroughly. Organoids derived from airway cells that can imitate the form and function of the lung ex vivo while allowing for experimental manipulation have given a new and intriguing model system for lung biology research [59].

Organoids demonstrate genetically identical profiles, molecular characteristics, and morphological features to the relevant patient tumour tissue during and after

long-term growth. Using organoids as lung cancer patient avatar models would be excellent for investigating the processes causing tumour recurrence after therapy and, as a result, assisting in the development of tailored medicine [60].

In vitro tissue culture or tumour spheroid culture using a three-dimensional culture system have been investigated as individualised models for lung cancer to predict response to anticancer therapy. However, they have limited growth and replication of the original tumour architecture. Lung cancer organoids (LCOs) have been cultured recently using the airway organoid method, which consistently retains their parental tissues' morphological and genetic characteristics. This LCOs may be used in patient-specific pharmacological trials and proof-of-concept studies on targeted therapy and resistance mechanisms [61]. The cancer organoid was also preserved tumorigenicity, as indicated by cytologic characteristics of malignancy, xenograft development, retention of mutations, copy number aberrations, and gene expression patterns by whole-exome and RNA sequencing between the organoid and matched parental tumour tissue. The establishment rate did not change significantly according to the tumour stage, location, or whether the material was acquired by biopsy or surgical excision [56].

Current Limitations and Future Directions

Organoids continue to be an imprecise approximation of real-life tissues. Organoids generated from ASCs contain various lung epithelial cell types, but organoids derived from PSCs contain mesenchymal cells [62]. Organoids in their current state, however, lack non-epithelial components. Apart from a fixed amount of mesenchyme-derived signalling molecules and extracellular matrix, functional connections between the epithelium and surrounding stromal cells are not reproduced. The interactions between the epithelium and non-epithelial parts are essential for controlling developmental processes and determining respiratory disease characteristics. Non-epithelial cells, including endothelium, smooth muscle, and immune cells, will eventually replicate the function of the actual organ [3]. Indeed, it has been demonstrated that adding mesenchymal cells into the culture improves the culture of alveolospheres [11].

Different organoids can be obtained from the same cell by altering the culture microenvironment [63]. This technique enables the development of organoids tailored to the research objective; however, it may affect the reproducibility of the organoids, resulting in skewed research results. The use of a stable ECM may provide a solution to this issue. Lung organoids are generally spherical and do not entirely replicate the lung's morphological structure. For instance, the branching airway is an essential component for lung air conduction. Thus more nutritional support optimised medium formulations for organoids in culture systems and extended culture periods may be required to overcome this issue [3]. Organoid morphogenesis may be used with bioengineering techniques such as 3D bioprinting or biomaterial scaffolds to construct architecturally complete organoids rapidly [64, 65].

In addition, more research is required to validate the interplay of many signalling pathways during an epithelial repair. Aside from the epithelium, this is a complicated interaction involving several cell types. The interaction of these cell types has yet to be comprehensively studied using lung organoids. Incorporating immune cells or vascular endothelial cells into lung organoids and co-culturing them to replicate in vitro microenvironment of epithelial regeneration could be used to better understand the link between cells and the ECM during epithelial regeneration [3].

Since SARS-CoV-2 infection is increasing globally, it is critical to developing new models utilising human disease-relevant cells to understand SARS-CoV-2 biology better and facilitate drug screening. The multi-tissue organ-on-a-chip platform (which incorporates various human-derived organoids) and lung organoids may be used to demonstrate the degenerative process of organs following SARS-CoV-2 infection, screen prospective medications, and develop and test vaccines for safety and efficacy [66–69]. The organoids are being utilised to study the novel virus and to imitate the symptoms of SARS-CoV-2 infection in humans (47. In conclusion, lung organoids coupled with various technologies may aid researchers in better understanding lung epithelial regeneration and improving therapeutic strategies to treat respiratory diseases.

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Chapter 4
Recent Advances in Brains Organoids: Opportunities and Challenges

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Abstract **Introduction**: Recent development in brain organoids has heightened the need for generating specific brain region-organoids. The directed and undirected neural fate of stem cells to generate brain-specific organoids are discussed, and here we summarised the leverage of the brain organoids over the animal models of neuroscience-related diseases. This chapter also discussed the current studies and their applications in autism spectrum disorders, epilepsy, Parkinson, and Alzheimer's disease paradigm to address the good use of brain organoids. The last part of the chapter summarised the challenges of using brain organoids in bioethical issues and the bio-plausibility context of brain organoid technology. **Methods**: The MEDLINE/PubMed database was used as a platform to search literature. The keywords used in the MEDLINE research were brain organoids, neurodevelopmental disorders, bioethics, biopausibility. In total, we found 64 articles that were recently published within 6 years interim up to August 2021 that focus on recent development in brain organoids, opportunities, and major challenges framework. **Results**: Rapid advances of brain organoids include the derivation to cortical-, cerebral-, midbrain-, forebrain-specific organoids and these brain regions are essential in better understanding the spectrum of neurodevelopment and neurodegenerative diseases. It is an attractive approach to overcome the limitation demonstrated in the current conventional model described in two-dimensional culture and animal models. **Conclusion**: The success protocol of derivation to generating specific regions of brain development may provide cues to rapid advances to understand neurodevelopmental disorders or psychiatric diseases. However, these advancements could eventually push the boundaries, and one should consider ethical issues and the limitation of using brain organoids as alternative methods to study brain development.

Keywords Brain organoids · Neurodevelopmental disorders · Bioethics · Biopausibility

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Abbreviations

iPSCs	Human pluripotent stem cells
NPCs	Neural progenitor cells
bFGF	Basic fibroblast growth factor
FGF	Fibroblast growth factor
SHH	Sonic hedgehog
RA	Retinoic acid
Wnt	Wingless/integrated
SMAD	SMA-decapentaplegic
FGF9	Fibroblast growth factor-9
FGF2	Fibroblast growth factor-2
CNTF	Ciliary neurotrophic factor
SDF1	Stromal cell derived factor-1
ROCK	Rho associated protein kinase
2D	Two dimensional
3D	Three dimensional
NDD	Neurodevelopmental disorder
ASD	Autism spectrum disorder
FOXG1	Forkhead box protein G1
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/caspase-9
TSC1	Tuberous sclerosis complex-1
TSC2	Tuberous sclerosis complex-2
PD	Parkinson disease
AD	Alzheimer disease
hMO	Human midbrain organoids
mDAN	Midbrain-specific dopaminergic neurons
AADC	1-Amino acid decarboxylase
TH	Tyrosine hydroxylase
DAT	Dopamine transporter
SMA	Spinal muscular atrophy

The Origin of Brain Organoids

The ideas of organoids development were branched out from the discovery of Takahashi and Yamanaka on the induced pluripotent stem cells (iPSC) in 2006. These cells can differentiate with the help of external and intrinsic factors introduced in the culture system. The advancement of this technology is leveraged into the development of a more sophisticated disease model. The generation of three-dimensional (3D) organoids and cocktails of differentiation factors make an alternative model much more dynamic and interesting. One of the most important events of 2009

was the first organoid developed for the small intestine by Sato et al. [1] and more subsequent organoids were developed after that, including the brain organoid model [2]. A considerable amount of literature has been published on the development of brain organoids. These studies include discoveries in neurodevelopment and neurodegenerative disease treatment strategies and glioblastoma using brain organoids as a platform for animal models. The development of brain organoids started from the cerebral organoids developed in 2013 [2]. Following that year, more protocols on the development of other brain organoids have been generated, such as to model related to the blood-brain barrier (BBB) [3], midbrain [4], hippocampus [5], cerebrum, cerebellum and forebrain [6] organoids have been successfully developed. Recent developments in brain organoids have heightened the need for generating specific brain region-organoids. The surge of this development has seen increasingly rapid advances to understand psychiatric disease [7], neurodevelopmental [8–10] and neurodegenerative [11, 12] disorders. These models become an attractive approach to overcome the limitation demonstrated in the current conventional model described in 2-dimensional culture (2D) and animal models.

Neural Fate

Two different approaches can guide the neural fate and generate brain organoids, (i) directed neural fate differentiation and (ii) undirected neural fate differentiation. Directed neural fate differentiation referring to the approach where the supplementation of gradient external morphogens is required to induce human pluripotent stem cells (iPSCs) to differentiate towards desired lineages in neurogenesis. Undirected neural fate differentiation relies fully on the intrinsic differentiation capabilities of the neural progenitor cells to differentiate towards its lineages naturally without external patterning factors being introduced. This section discussed the differentiation protocols reported in published studies the significance of morphogens until now.

iPSC research was pioneered by Professor Shinya Yamanaka and his doctoral student, Masayo Takahashi [13]. The iPSCs technology holds great promise and enabled unprecedented opportunities to study the most complex organs in the human body. Human iPSCs are pluripotent stem cells generated directly from a somatic cell, including the neural progenitor cells (NPCs). Neural rosette is the term that describing a group of polarised NPCs generated by the adherent culture of the embryoid body. Neural rosette generated in vitro resembles the early neural tube when spreading onto an adhesive substrate in the presence of a basic fibroblast growth factor (bFGF). Their morphology from tightly packed epithelial cells changed into elongate neural stem cells. The establishment of a neural tube radially organised around a lumen leads to the dishing out of the human mini-brain, called brain organoids [2]. With the knowledge of developmental biology, the forebrain gives rise to the neocortex, hippocampus and ventral telencephalic (amygdala and hypothalamus); midbrain

gives rise to tectum; hindbrain gives rise to the cerebellum, pons, medulla, and brainstem [2, 6, 8, 14]. It holds a major milestone of human brain development in vitro. This technology can mimic neurogenesis in vivo, such as forming regional neuronal circuitry by integrating glial cells into brain organoids and forming a neural network as in the human brain.

The human brain organoids protocol was established by Lancaster et al. and further modified by Steven A Sloan in 2018 [8, 15] to produce brain organoids that resemble human brain regions. The growth of human cerebral organoids can be achieved by implanting an embryoid body in a Matrigel matrix to anchor the cells on a surface, assist tissue formation, and use a spinning mini-bioreactor method to increase the gaseous exchange and nutrient distribution to the organoids. The current protocol can well-maintain the apical-basal polarity, interkinetic nuclear migration, division modes of neural stem cells, and the pattern of neuronal migration [8, 15].

Early organoid differentiation protocols largely depend on the intrinsic signalling and self-assembly of stem cells, termed undirected neural fate differentiation. During neurogenesis, stem cells switch from symmetric divisions that aim to increase their numbers to asymmetric division to give rise to the NPCs capable of self-renewing and more differentiated lineage-specific cell types such as neurons and intermediate progenitors [2]. These cells then migrate outward and self-organised into stratified structures such as the three layers of the medulla, the seven layers of optic tectum and the six layers of the cerebral cortex. The neural cells can self-organise into neural tissue from the pluripotent stem cells. Koo suggested that the adhesion proteins on a cell surface play an important role in driving the autonomous cell sorting of the specific cell, which leads to the clustering and layering of the cells [16]. Besides, a study suggested that cell fate will decide the spatial restriction of its offspring cells from the progenitor; this may contribute to self-organisation [2]. The self-organisation of organoids is explained by the adhesion proteins, spatially restricted cell-fate, and the contractile cytoskeleton. The contractile cytoskeleton works together with cells to generate intrinsic tissue-scene, tissue shape, and organisation processes [17].

However, region-specific differentiation factors or morphogens can induce the growth of neural stem cells to desired lineages in vitro (summarised in Fig. 4.1). Axes are established through the concentration gradient of the morphogen introduced, such as sonic hedgehog-wingless/integrated-bone morphogenetic protein (SHH-WNT-BMP) axis contribute to the dorsal-ventral axis (roof, alar, basal and floor plate) and the rostral-caudal axis (tel-, di-, mes- and rhomb- encephalon and spinal cord) influenced by the differentiation factors such as retinoic acid (RA) and fibroblast growth factor (FGF). Further, WNT signalling is stage-specific and plays an important role in body axis determination and cell fate patterning [18]. Inhibition of WNT signalling promotes neuronal induction and neuroectodermal differentiation during the early stage embryoid body, whereas activation of WNT signalling promotes the mesoderm differentiation [6]. The WNT signalling has therefore been used in combination with SMAD inhibition (or dual-SMAD inhibition) to promote the production of neuroepithelial-like organoids [19, 20] and also found to reduce cell death significantly [6].

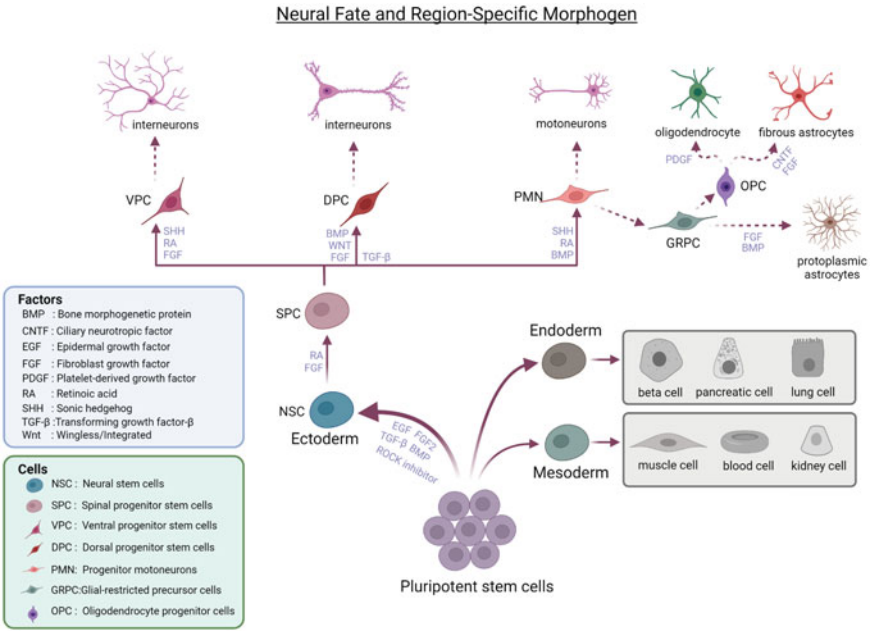

Fig. 4.1 Neural fate and its region-specific morphogen

Collectively, hypothalamic-like organoids could generate by SMAD inhibition followed by the addition of WNT-3A, SHH during day 3 to day 7 and FGF-2 and CNTF after day 7 [6]. Muguruma and colleagues generated Cerebellar-like organoids by cocktails of SMAD inhibition and ROCK inhibitor and sequential addition of FGF9 and SDF1 in neurobasal medium [21, 22]. The region-specific organoids generated in vitro could recapitulate the molecular, cellular, and cytoarchitecture of the human brain regions. In brief, inhibition of the SMAD signalling pathway is commonly used to inhibit mesoderm formation and endoderm, followed by using specific morphogens at the early differentiation stage to act as neural fate-specifying molecules to induce into desired neural linages with minimal heterogeneity. These morphogens are removed or minimised after successful patterning, and subsequent differentiation follows intrinsically programmed cell fate. The neural fate differentiation can sometimes generate brain miniatures with relatively consistent cell types [1] and exhibit less variation across batches [23].

To address the possibility of the biological and functional of directed neural fate differentiated organoids, Steven and his colleagues resembled the human cerebrum by inducing pallium-like organoid and subpallium-like organoid, then fused them to form an assembloid, a combination of organoids resembling distinct areas of the brain as one compartment that can model aspects of interactions that occur between regions in the human brain [15]. From the study, Sloan et al. found that fluorescence-labelled inhibitory neurons successfully migrated from the subpallium-like part of

the assembloid to the pallium-like part of the assembloid, consistent as interneurons generated from the ventral domain will migrate towards the dorsal domain in vivo [15, 23]. A more substantial approach to characterise differentiated organoids, Birey's research group performed electrophysiological characterisation of the forebrain assembloid revealed that microcircuits formed between the synaptic connection of interneurons and local excitatory neurons were well-function [24]. Furthermore, the functional astrocytes present in cortical-like spheroids [23] and oligodendrocytes in oligocortical spheroids [25] were also workable to generate brain organoid technology. Overall, these findings hold the major milestones to decipher the secret of the human brain in the future.

Brain Organoids Derived from Human and Animal Models

In this section, the comparison between brain organoids derived from human and animal models, as a model for biomedical research, and to what extend both models have interconnected each other, the strength and weaknesses of the model are discussed. It has been shown that interspecies comparison at the cellular and molecular level of cerebral organoids derived from human, chimpanzees and orangutan iPSCs, revealed that the cytoarchitecture, cell-type composition and neurogenic gene expression of humans and chimpanzees are remarkably similar to each other, although there is a significant difference between human and orangutan [26]. Mora-Bermudez et al. demonstrated that the human organoids showed lengthening of progenitor replication during prometaphase-metaphase that may have consequences for human neocortex evolution [26]. Mora concluded that the human brain could be viewed as a triple scaled-up primate brain, chimpanzee's brain, our closest relative [26].

Several comparative analyses were demonstrated at the transcriptome level [27], epigenetic level [28] and epitranscriptomic level [29]. These studies showed high similarities between human brain organoids and the human brain at a different level of analysis. Along the same lines, Behjati et al. revealed that brain organoids showed genetically and morphologically stability over long-term culture[30]. Camp et al. also found that the protein expression and cellular diversity in the organoids and brain samples are highly similar, although several differences are observed [27]. Pollen performed single-cell RNA-sequencing, and gene-network analysis revealed upregulated gene expression in the human brain compared to macaque. Following this, they were also found that those genes were upregulated in human brain organoids compared to chimpanzee organoids [31]. These data suggest that brain organoids derived from humans are significantly different compared to animal models but highly similar to the human brain at the genetic level; hence, the brain organoid model might be an ideal study model as the alternative for animal models.

Looking at the cellular level, the division of neural stem cells' patterns are different from human and mice. Most of the radial glial cells were inhabited in the ventricular zone of the mouse developing neocortex, but in humans, ventricular radial glial cells and outer radial glial cells were found in the enlarged outer subventricular

zone neocortex. Thus, this explains why the division of patterns of neural stem cells exhibit major differences between mice and humans [32]. Kelava and Lancaster argued that stem cell models take five days in murine and twenty days in humans for neuronal differentiation, suggesting that organoids are species-specific. Thus, the generation of brain organoids has species-specific intrinsic timing [33]. The use of immunodeficient rodent models poorly represents the biological and functional microenvironment of human cancer. Thus, organoid technology might be the alternative for its representative. Unlike nearly all the animal models, most of them failed to recapitulate the three-dimensional (3D) biological structures in humans, especially in the tumour microenvironment. In short, animal brain models are different at the genetic level and cellular levels.

Recently, considerable evidence has accumulated that brain organoids are safe and gain functionality after transplantation to host animals. Hans Clevers presented the data that brain organoids generated in their laboratory survive after transplanted to mice, whereas cells derived from grown cultures show indications of inducing tumour formation [34]. Mansour and his colleagues further supported Clevers's notion by transplanting whole-brain organoids into an adult mouse. The study reported successful transplantation, showing brain organoids' anatomic and functional integration with the host environment [35]. Much of the literature concerns the feasibility of long-term culture of the brain organoid. Investigation of Qian and his research team corroborates the idea of putting the brain organoids in long-term culture and successfully generating all six cortical layers using an improved spinning mini bioreactor [6]. This study has further improved our knowledge and technology in brain organoids. Indeed, organoids technology own the advantage over animal models as it uses human tissues as the source. Studies demonstrated that animal models failed to provide conclusive results due to physiologically differences when compared to humans. For instance, a meta-analysis study illustrated that the percentage of the failures of drugs toxicity articulated from animal models had been failed to predict the drug toxicity in humans in pre-clinical and clinical trials was 88% and 88.3%, respectively [36].

The brain organoids model still has several limitations. For instance, the current brain organoids lack the immune system, although this might be overcome by establishing a co-culture of brain organoids and immune cells. The co-culture protocols are maybe unlikely to recapitulate the detailed immune responses underlying the animal models. Therefore, it seems that animal models and organoid approaches should be interdependent rather than in opposition. Much of the available literature on brain organoids deals with the lack of a vascular system. However, Mansour et al., in 2018, successfully developed a vascularised brain organoid by experimenting with transplanting the human brain organoids directly into adult mouse brains [35]. The graft functionality was assessed using several confirmatory methods, including immunofluorescent imaging, electrophysiology, optogenetic stimulation and Barnes maze behavioural test. The integration and function of blood vessels with the host brain were demonstrated after transplantation [35]. Later in the following year, in 2019, Wimmer et al. developed human blood vessels organoids to model diabetic vasculopathy, and it did recapitulate the structure and function of human blood vessels

and proposed an amenable system for modelling [37]. Although it is not directly involved in brain organoids, it sheds light on more research to make a dynamic vascularised brain organoid soon.

The Opportunities of Using Brain Organoids

Brain organoids are self-organised, 3D-aggregates derived from hPSC or iPSC, which comprises cell types and cytoarchitectures resembling the human embryonic brain [14, 38]. Brain organoids were proposed to understand the pathogenetic mechanism of disease associated with monogenic and polygenic genomic alteration due to their ability to preserve the human genomic context. Brain organoids also help capture complex phenotypes on different neuronal networks, organ morphogenesis, and tissue architecture. Meanwhile, species-specific developmental events can be embodied in vitro. For example, the duration of the neurogenic period, rate and pattern of cell migration and cell cycle dynamic events can be recapitulated using brain organoids [38]. There are two types of brain organoids: -the cerebral organoids (also known as the whole-brain organoids) and the directed regional brain organoids. Cerebral organoids can be formed from the ability of the pluripotent stem cells to self-organise and self-pattern. Here in this section, we discussed the application of brain organoids in neurodevelopmental disorders, i.e., epilepsy and autism spectrum disorder and some neurological diseases such as Alzheimer's disease and Parkinson's disease.

Neurodevelopmental Diseases

Brain organoids can be used as a model system for neurodevelopmental disorders (NDD) such as autism spectrum disorders (ASD) and epilepsy. Patients with ASD are associated with difficulty in social interaction and communication, restricted and repetitive patterns of behaviour activities or interests. Genetic defect at the early stage of embryonic or foetal affected neurogenesis and cortical lamination, which have been one point of convergence for ASD. Due to the lack of material and ethical constraints on using human embryos for research, brain organoids will be an alternative option to study prenatal brain development. Brain organoids give a high resemblance to embryonic brain tissue. Most of the cell types present in the embryonic brains can be found in brain organoids in an orientation that can be found similar in vivo with the exhibition of similar-like behaviours [39]. It has been demonstrated that increased over-expression of a transcription factor, FOXG1 in cerebral organoids, results in markedly increased production of inhibitory neurons and serves as a therapeutic target for idiopathic ASD. Excessive GABAergic neurons are believed to become important mechanisms involved in ASD [8, 40]. A further imbalance between excitatory and inhibitory ratios in ASD could contribute to its

pathogenesis [41]. Subsequent years later, FOXG1 is a potential molecular signature for idiopathic ASD [39].

Like the ASD paradigm approach, epilepsy also can benefit from using brain organoids to examine the genetic defects during its development. Epilepsy is a chronic neurological condition of unprovoked and recurrent seizures caused by neuronal hyperactivity [42]. Few other syndromes related to the brain neurodevelopment disorder of epilepsy such as tuberous sclerosis, Rett, Timothy syndromes. These complex interactions between epilepsy and subsequently associated syndromes can be established using brain organoids. To further extrapolate the dynamic of brain organoids with the CRISPR/Cas9 gene-editing method, 'two-hit' hypotheses' experimental design of rare and multi-systemic genetic diseases can be addressed. For instance, tuberous sclerosis presented with homozygous loss of tuberous sclerosis complex 1 (TSC1) and 2 (TSC2) in cortical development, which leads to the disruption of the development suppression of mechanistic target of rapamycin complex 1 (mTORC1) signalling [43, 44]. The mutation in the TSC1 or TSC2 genes and its role in regulating tuberous sclerosis disease's pathology can be explored. Evidence suggests that the mutation in the CACNA1C gene is one of the hallmarks of Timothy syndrome, which is described as a rare genetic disorder that primarily affects the heart and affected brain development and other organs. The CACNA1C gene encodes for the L-type calcium channel Cav1.2 α subunit, and this gene mutation led to the production of abnormal inhibitory neurons [45]. The imbalance of excitation and inhibition ratio affected the flow of brain regulation [46]. A significant study conducted by Birey et al. showed that fused organoids as an approach for modelling neuronal circuits with distinct brain regions using in vitro methods [38].

Alzheimer's Disease (AD)

Due to the limitation in the 2D cell culture model system, brain organoids modelling has attracted considerable interest to model AD. AD is a neurodegenerative disease that is advanced age-related, characterised by psychiatric and cognitive symptoms like behavioural abnormalities, circadian rhythms, memory, cognitive impairments, and sensory disturbances. AD causes dementia in the elderly and has affected over 50 million people worldwide [46, 47]. The 3D system in vitro of AD derived brain organoids are established and successfully use as platform strategies for drug screening treatment. The pathophysiological AD-like features such as endosome abnormalities, tau hyper-phosphorylation and amyloid abnormalities can be exhibited through organoids derived from the human pluripotent stem cells of familiar AD patients [47]. Brain organoids also help in the in vitro replication of Alzheimer's disease's molecular determinants like tau pathology, Aβ and synapses dysfunction. The production of toxic Aβ can also be inhibited partially through the treatment with ɣ-secretase or β-secretase inhibitor compounds and suggests the Aβ-driven tauopathy theory and reduce the hyper-phosphorylation of tau proteins [46].

Parkinson's Disease (PD)

A growing body of published work provides evidence of the success of brain organoids to model the PD paradigm. PD possesses motor symptoms such as tremor, rigidity and psychiatric symptoms like depression, apathy and executive dysfunction [48]. The midbrain-specific organoids play an important role to illustrate human brain development associated with PD pathophysiology and the disease modelling as implied by Smits et al. and Kim et al. Owing to model human midbrain organoid (hMO), Smits et al. observed the presence of PD relevant phenotypes that have been shown significant reduction the number of midbrain-specific dopaminergic neurons (mDAN) in the disease-associated G2019S mutation of the LRRK2 gene. This striking reduction was reported being found in both genetically modified and hMO. To further explain the dynamic of hMO, the dopaminergic network complexity in patient-derived TH-positive neurons were found significantly reduced. This reduction is explained by evaluating the number of branching and dendrite bifurcation points of the mDANs [49]. Kim et al. also found out that the neurite length of the mDANs in the mutated LRRK2-G2019S organoids decreased compared to mDANs of the control organoids [12]. The expression level of mDANs-specific markers such as aromatic l-amino acid decarboxylase (AADC), TH and dopamine transporter (DAT) can also be captured by these human midbrain organoids [49]. Taken together, these findings of PD associated events in hMO indicated that brain organoids could be used as the therapeutic strategies for treating Parkinson's disease. Table 4.1 summarises the type of brain organoid used in current literature associated with neurodevelopmental syndromes and neurological diseases.

The Challenges of Using Brain Organoids

Bioethics

Organoids technology has helped biomedical research take a step forward in personalised medicine, and later, the main goal is to progress in transplantation medicine. However, some ethical issues have arisen concerning the origin of the cells used to produce organoids (e.g., human embryos) and their properties. For example, brain organoids have been created to overlook ethical issues to neuroscientists, stem-cell biologists, ethicists, and philosophers, since Lancaster and Knoblich started using induced pluripotent stem cells from human adult skin cells to create *in-dish* mini-brain to the model of microcephaly [2]. The brain organoids also exhibit neural connections and electrical activity [55–57] like a human brain, although the neural connections and electrical activity seen in the brain organoids models may not represent consciousness. However, this has alarmed ethicists about its ethicality, whether the well-developed organoids could generate consciousness, latter, could store and

Table 4.1 Type of brain organoids models and its association with neurodevelopment and neurological diseases

Type of brain organoids	Type of disease	Description	Author (s)
Cortical organoids	Aicardi-Goutières syndrome (AGS)	Three prime repair exonuclease 1 (TREX1)-deficient cortical organoid was developed to assess if the syndrome is associated with the microcephalic-like characteristics. This specific organoid was developed as AGS was highly associated with the deficiency of TREX1, as reported in microcephaly previously	Thomas et al. [50]
	Autism spectrum disorder (ASD)	Organoids derived from iPSC got from the patients and their parents to see the association of FOXG1 in increasing GABAergic neurons that leads to the disease	Mariani et al. [40]
Cerebral organoids	Microcephaly	They investigated the tendency of the Zika virus to cause microcephaly in the organoid model they developed	Cugola et al. [51]
		The organoids developed have had some aspect in microcephaly to figure out if it is an appropriate model to replace human cells and animal models	Lancaster et al. [8]
	'Alzheimer's disease (AD)	Organoids developed have been used to study the electrophysiological activity following AD. From the findings, they claimed that their model is reliable to understand the mechanism of AD instead of using in vitro and transgenic AD mouse models	Ghatak et al. [11]
	Autism spectrum disorder (ASD)	The organoids developed from iPSCs got from the patients to investigate the transcriptome analysis of ASD-related gene, *CDH8,* in various brain-related diseases	Wang et al. [52]

(continued)

Table 4.1 (continued)

Type of brain organoids	Type of disease	Description	Author (s)
	Miller-Dieker syndrome (MDS)	The organoid has been developed to study the mechanism associated with MDS, including lissencephaly, which the environment is difficult to develop in an animal model	Bershteyn et al. [9]
	Sandhoff disease	The authors modified the previous protocol of cerebral organoids development to fit the characteristics of the disease they were interested in	Allende et al. [53]
Midbrain organoids	'Parkinson's disease (PD)	The pair of isogenic midbrain organoids developed, which differ at LRRK2 locus, associated with the formation of PD, have shown a promising representative to study the behaviour of the gene and its effect on the pathophysiology of PD	Kim et al. [12]
Forebrain organoids	Miller-Dieker syndrome (MDS)	The organoids were used to study the effect of alteration of microtubule network of ventricular radial glia cells (vRGCs) on the N-cadherin/β-cadherin/Wnt signalling following the disorder	Iefremova et al. [10]
Spinal organoids	Spinal muscular atrophy (SMA)		Hor et al. [54]

retrieve a memory.Theoretically, brain organoids have only about one hundred thousand neurons compared to the 86 billion in a full-sized brain [58]. Owing to this constraint, the possibility of brain organoids gaining consciousness or a higher-order property, at least in the current stage, seems highly remoted. The closer the human capability to gets to a functioning human brain, the more the ethical problems it becomes, such as how the consent should be performed, stewardship of the generated brain organoids, who should deserve the ownership of the brain organoids, the data ownership that could reveal sensitive information such as individual memories or personalised genetic status, and post-research handling of the brain organoids.

Research in interspecies chimaeras has shown that they could produce rat-mice chimaeras by injecting rat pluripotent stem cells into mouse embryos [59]. The same approach could likely produce human-animal chimaeras. With these chimaeras being

produced, these consequences might lead to catastrophic ethical issues pertaining to human-animal chimaeras. At that time point of time, how do we define the boundaries of human identity and animals? Another striking question that might be raised in producing a human organ such as the heart or pancreas in a pig's body is acceptable, for instance, but not the growing neural tissue in animals from human cells? A more comprehensive study would include all the possibilities if we are keen to venture into this futuristic transplantation medicine.

Other than bioethics, philosophical issues also need to be considered, such as the death-life issue. Advancing brain organoid models might challenge our pre-existing understanding of life and death and the legal definitions of death. In the early 1960s, a completely and irreversibly ceased brain, so-called brain death, could be declared dead, even if their heart is still beating. Therefore, we sought to understand the implications of what if the ceased brain function might no longer be permanent and irreversible, or even brain transplantation could be performed, and we might need to rethink and redefine the death of a human being. As a result, religious fanaticism is likely to disapprove of this research. Like what has happened during introducing gene-editing technique, CRISPR, all these for them, seems to be "playing God" and should be halted even before it starts.

Julian Koplin and Julian Savulescu [60], the research fellow with the Biomedical Ethics Research Group at the University of Melbourne and Uehiro chair in practical ethics at the University of Oxford, should introduce some moral limits to research with brain organoids. Both of them proposed that brain organoids search should proceed only if (i) the aim of the research is outweighed the expected costs (including harms), (ii) the research cannot be conducted using non-conscious or non-sentient organoids, (iii) research only use the minimum number of organoids to answer the research questions, (iv) the organoids used do not have a higher potential capacity of harm than is necessary to achieve the research objectives, (v) the research should minimise possible harm and (vi) the research would not inflict severe long-term suffering, to achieve some critically important purpose. The research restrictions of using brain organoids have been summarised in Table 4.2.

Although brain organoids could raise many ethical and philosophical concerns, these conundrums should not be a barrier and hold back brain research. If brain organoids show any sentience one day, an ethical discussion on their clinical and research use and practice would be necessary. Regarding human brain research, we still cannot decode the mysteries about psychiatric and neurological diseases, which have long remained elusive. Brain organoids can be considered a more ethical research methodology, supported 3Rs (Replacement, Reduction and Refinement) method. In fact, no living life is being destroyed, damaged, or put at risk in the research involving organoid models.

Table 4.2 Brain organoids models and its potential research restrictions

Brain organoids models	Research restrictions
Non-conscious	1. Research should be aligned to the current existing guidelines framework as stated by the International Society for Stem Cell Research (ISSCR) and International Society for Biological and Environmental Repositories (ISBER)
Conscious/potentially conscious	2. Besides the above, Julian Koplin and Julian Savulescu suggest that brain organoids should be subject to the following restrictions: 1. The research aims to outweigh the expected costs (including harms) 2. The research cannot be conducted using non-conscious or non-sentient organoids 3. The research only uses the minimum number of organoids to answer the research questions 4. The organoids used do not have a higher potential capacity of harm than is necessary to achieve the research objectives 5. The research should minimise possible harm 6. The research would not inflict severe long-term suffering, to achieve some critically important purpose

Biopausibility

To further advance brain organoid technology, extra-cerebral origin cells such as microglia are required. It may have a key developmental role in the brain, particularly in synaptic pruning that occurs between early childhood and adulthood. Abud and his colleagues had made this workable as they have demonstrated the introduction of microglial derived from iPSCs into the brain model [61]. This approach allowed, for the first time in human history, to study the pruning of human synapses in vitro, and this system might have a great promise for the human to study more complex phenomena such as neurodegenerative, neuropsychiatric, neuronal cell-cell interactions, neuroplasticity, neural network, neuro-electrophysiological circuits, so on and so forth. Paola Arlotta suggested that even though's human organoids technologies can generate active neurons and functional neuronal circuits, none of them can recapitulate the anatomical organisation, electrophysiological functions, and connectivity patterns of the endogenous brain [62].

The advancement of brain organoids has experienced unprecedented growth over the past few years and could eventually push these boundaries—for example, by wiring brain organoids to muscle tissue [63], by connecting brain organoids to controllable robotic "bodies" or by implanting human brain organoids into non-human 'animals' brains [35]. Recent development in brain organoids created "photosensitive" brain organoids, which feature rudimentary eyes and display neural activity when light is triggered [64] form of brain organoids expressing biological markers found in all six cortical layers has been demonstrated in recent studies [6]. None of them forms the six distinct layers seen in the human cortex. The brain cells were endowed with regenerative capabilities, in which the cells did not grow and

expand in the dish easily. Indeed, the elementary level to understand how the human brain develops and functions, as the brain is formed largely in utero, is still lacking. There are obvious ethical considerations that are limiting access to the human brain for research. These difficulties have hampered human efforts to decipher the secrets of the human brain.

Due to inaccessibility to live human brain tissues, animal model organisms, especially mice, have been an option to examine the brain. Unfortunately, there are huge differences between the development of the rat's brain and that human's brain, as we mentioned earlier. Collectively, current limitations of brain organoids are restricted by (i) survival, (ii) oxygen and nutrients distribution, (iii) inter-batch heterogeneity, (iv) tissue architecture, (v) gliogenesis and (vi) neuronal activity. Table 4.3 summarises the current limitations of brain organoids.

Table 4.3 The current limitations of brain organoids

Current limitations of brain organoids model	Comments
Survival	Brain organoid models can now survive for long-whole culture up to 1 year. However, there is a need to further long-term maintenance of brain organoids over one year to study the later stage of its maturation. Even 'today's brain organoids have been transplanted into an adult mouse, found that it promotes cell maturation, survival, and vascularisation of the brain organoids
Oxygen and nutrients distribution	Although several techniques developed such as agitation in an orbital shaker, spinning bioreactor, gas permeable dishes, on-chip method, hyperoxia culture environment, and organoid slicing method help provide nutrients and oxygenation to the brain organoids have resulted in prolonged survival and prevent the necrotic core formation. However, no functional vascularisation was demonstrated in vitro
Inter-batch heterogeneity	Reproducibility of brain organoids has been reported as "batch-effect", which is more pronounced in directed neural fate differentiation over the undirected neural fate differentiation. Thus, initial key patterning events need to be identified to control the homogeneity between batches of culture

(continued)

Table 4.3 (continued)

Current limitations of brain organoids model	Comments
Tissue architecture	Current brain organoids succeed to establish the deep layer and the upper layer of neurons, but the reassembly of the in vivo fully organised six-layer cerebral cortex cytoarchitecture remains rudimentary. Also, current organoid protocols have limiting organoid size and complexity, lack of endothelial cells, immune cells that contribute to the microenvironment of the brain
Gliogenesis	The complex structure comprises glial subpopulations all in the same brain organoid model to understand the functionality of neuron-glial interaction. Besides, gyrification, forming the characteristic folds of the cerebral cortex, and the formation of white matter tracts are missing in the current brain organoids
Neuronal activity	Multi-electrode arrays have demonstrated synchronised oscillatory network events in the brain organoid model. Hence further maturation of brain organoids might generate well-established connectome and network-based activity models. Besides, innervating of the peripheral nervous system is missing in current brain organoid models

Conclusion

Brain organoids are widely used in the application for neurodevelopmental disorders or neurodegenerative disorders. The advancement of brain region-specific organoids could help us study brain development and model human neurological disorders from better perspectives. However, one should consider ethical issues and abide by the law when conducting such advanced experiments. Unlike the brain, not much application of spinal cord organoids has been reported until now. However, the effort towards that has been seen recently. One of the earlier spinal cord organoids applications can be seen in the study conducted by Hor et al. to investigate the behaviour of motoneurons following spinal muscular atrophy (SMA) [54].

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Chapter 5
Toward Understanding Neurodegeneration Using Brain Organoids

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Abstract **Introduction**: With the advancement of pluripotent stem cells (PSCs), several brain regions have been modelled through brain organoids that resemble their in vivo counterparts of the human brain in terms of cellular heterogeneity and gene expression. The models can be used for molecular pathogenesis studies of neurodegenerative diseases and can be combined with many recent technologies such as optogenetics, CRISPR/cas9, patch-clamp, or on-a-chip system to create more precise models of brain development and diseases. Moreover, personalised organoids derived from patient-specific induced pluripotent stem cells (iPSCs) can also be used to develop personalised treatment. This chapter introduces the principles of brain organoid formation and the potential uses of brain organoids for modelling neurodegenerative diseases, drug development, and personalised medicine. **Methods**: We performed a literature review in PubMed (https://www.pubmed.ncbi.nlm.nih.gov) using the keywords brain organoids; neural differentiation; neurodegeneration; personalised medicine. **Conclusions**: Personalised brain organoids, which can be derived by several approaches and coupled with genome editing such as CRISPR-Cas9, have proved to be powerful tools for in vitro studies of early human brain development and pathogenesis. Future treatment of incurable neurodegenerative disorders should ideally be tailored to individual patients to obtain optimal efficacy. To this end, using in vitro patient-specific models of neuroectodermal tissues will allow for such customised treatment personalised medicine.

Keywords Brain organoid formation · Human pluripotent stem cells · Neurodegeneration · Personalised medicine

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Abbreviations

2D	2 Dimensions
3D	3 Dimensions
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
APP	Amyloid precursor protein
Aβ	Amyloid β
COVID-19	Coronavirus disease of 2019
CRISPR	Clustered regularly interspaced short palindromic repeats
EGF	Epidermal growth factor
ESCs	Embryonic stem cells
FAD	Familial Alzheimer's disease
FGF-2	Fibroblast growth factor-2
HSV-1	Herpes simplex virus type 1
iPSCs	Induced pluripotent stem cells
LRRK2	Leucine-rich repeat kinase 2
mDAns	Midbrain dopaminergic neurons
PD	Parkinson's disease
PLGA	Poly(lactide-co-glycolide) copolymer
PSCs	Pluripotent stem cells
PSEN1	Presenilin-1
PSEN2	Preselinlin-2
PTEN	Phosphatase and tensin homolog
SFEBq	Serum-free culture of embryoid body-like quick-aggregation
SMAD	Mothers against decapentaplegic
SNO	Sliced neocortical organoid
SOD1	Superoxide dismutase type 1
TNF-α	Tumor necrosis factor-α
WNT	Wingless and Int-1

Introduction

The neocortex of the human brain is populated by a great diversity of neuronal cell types. It is responsible for the higher cognitive functions which cannot be found in any other species. Understanding human brain development under normal and disease conditions is a major goal to discover new therapeutic approaches for the nervous system. The use of animal cells/models for neurobiology research is a longstanding practice. However, animal cells/models do not reflect but only share some features of human diseases. Accessibility to human brain tissue is limited due to ethical concerns associated with their origin. The establishment of in vitro cultures of neuronal and immortalised neuroblastoma cells overcomes this limitation. Although both neuronal

cell culture systems are regularly used in neurobiology research, the conventional 2D culture systems do not recapitulate the brain microenvironment, complexity of neural tissue, and disease phenotypes such as abnormal protein aggregation. Another limitation is that they contain only one cell type and do not have cell-cell interaction between cell types.

In the pre-organoid era, the ex vivo brain tissue slice culture, known as an organotypic culture, has been used in neurobiology research (Fig. 5.1). Many different brain regions can be sliced and cultured for weeks to months. They have been used as a model to study the brain microenvironment, which resembles in vivo conditions of the precision area of the brain [1]. Unlike neuronal cell culture, organotypic culture maintains cytoarchitecture and the microenvironment of the brain. Therefore, organotypic culture is a functional tool for neurobiology research and drug screening.

On the other hand, neural precursor cells were isolated from various human brain regions, cultured in vitro, and formed free-floating hollow clusters of neural stem/progenitor cells called "neurospheres" [2, 3]. Neurospheres are heterogeneous

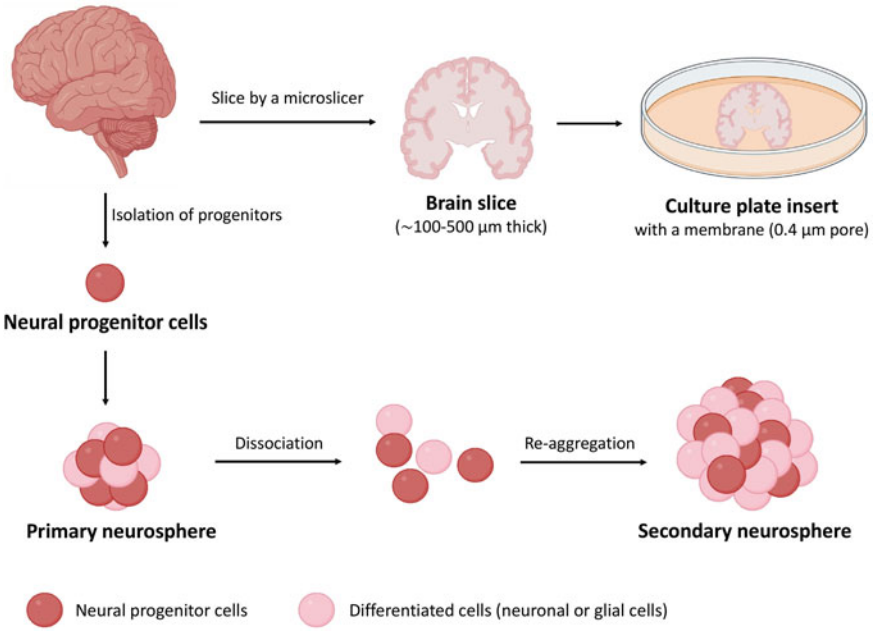

Fig. 5.1 Conventional culture methodologies for brain cells and tissues include ex vivo organotypic culture (top) and neurosphere culture (bottom). For organotypic culture, brain slices at the thickness of 100–500 µm can be prepared from relevant brain tissues using a vibratome and placed in an insert with 0.4-µm semipermeable pores. For neurosphere culture, different brain areas, such as the dentate gyrus and subventricular zone, can be isolated to derive neural progenitor cells. Upon expanding neural progenitor cells, they can further self-renew to give rise to identical progenitors or differentiate to produce neurons and glial cells. Expansion of neurospheres can be performed by dissociation and re-aggregation of the neurospheres (Made in ©BioRender—https://www.biorender.com)

and comprise several hundred astrocytes, neurons, and neural stem/progenitor cells with specific characteristics based on their region of origin [4]. Neurospheres can be propagated by mechanical chopping or dissociation, overcoming the limitation of organotypic culture. Although neurospheres have been known as a tool to study neurogenesis and model for the early development of the human brain, it has lower complexity and less structural organisation than organoids.

The advanced development of human pluripotent stem cells (PSCs), both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have provided a platform of model systems for understanding human biology, physiology, development, and diseases. Treatment with essential growth factors promotes human PSCs differentiation into specific cell lineage. Many groups have developed neural induction protocols to drive hPSCs to become neural cell types in 2D and 3D cultures. A very early method to generate pre-rosette neural stem cells in neurospheres (termed EZ spheres) has been developed by lifting hPSCs colonies and cultured in a neural stem cell medium with a high concentration of EGF and FGF-2 [5]. The EZ spheres can form neural rosettes and further differentiate into several types of neural lineages. Chandrasekaran and colleagues compared the efficiency to generate neural stem/progenitor cells from hPSCs between 2D induction and 3D induction methods. A higher number of neurons with longer neurites were observed in 3D neural induction, suggesting a superior way to generate forebrain cortical neurons from hPSCs [6].

An organoid refers to 3D suspension culture techniques to generate an organised organ/tissue resembling various in vivo-like cellular architecture features in a dish. Fatehulla and colleagues defined organoid as "an in vitro 3D cellular cluster derived exclusively from primary tissue, embryonic stem cells, or induced pluripotent stem cells, capable of self-renewal and self-organisation, and exhibiting similar organ functionality as the tissue of origin" [7]. Therefore, an organoid is technically different from a neurosphere, which refers to an aggregate of neural derivatives without any cytoarchitecture and morphogenesis found in the brain. By combining PSCs technology and differentiating iPSCs into neuronal cells, an innovational study by Lancaster and colleagues has shown a culture system to generate cerebral cortex-like organoids from human PSCs derived from a patient with microcephaly [8]. Brain organoid or cerebral organoid composed of progenitor, neuronal and glial cells and resemble the human fetal brain [8–10]. Since then, effective protocols for brain organoids cultures have been established. Exogenous cues such as Wingless and Int-1 (WNT) inhibitor and Mothers against decapentaplegic (SMAD) inhibitor were used to mimic the endogenous developing pattern and direct neural induction in a high consistency of brain organoid generation. Although the short-term culture brain organoids reflect the immature state of the brain, neurons in brain organoids begin to mature after 60 days in culture and show spontaneous excitatory post-synaptic currents after 120 days in culture [11, 12].

To date, several brain regions, e.g., thalamus [13], midbrain [9, 14, 15], pituitary gland [16], cerebellum [17, 18], and brainstem [19], have been modelled using brain organoids. As the brain organoids have some main features of the human brain, e.g., cellular distribution and organisation, electrophysiological functions, and neural

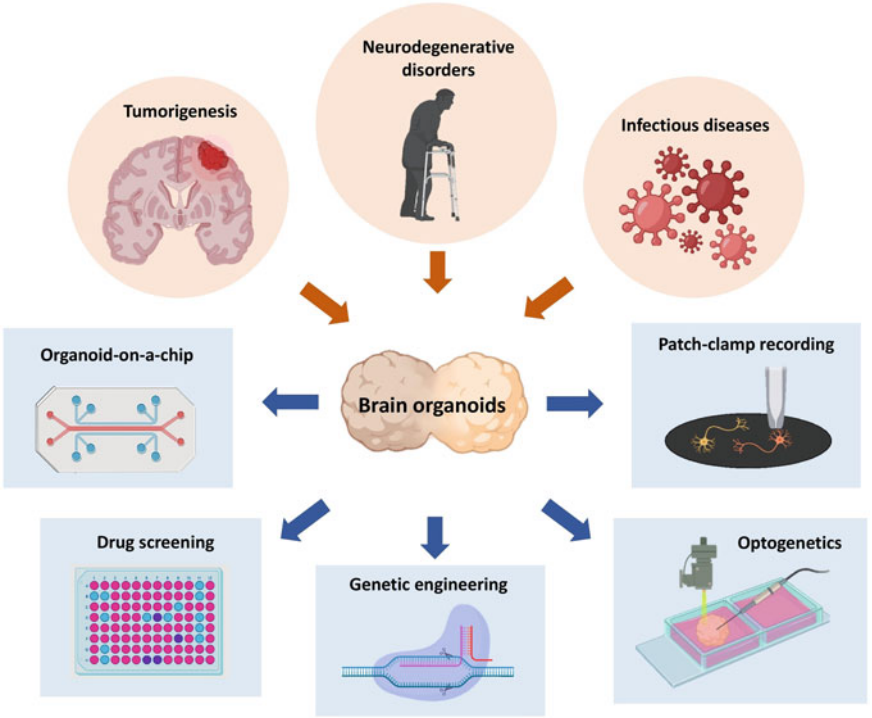

Fig. 5.2 Brain organoids have revolutionised research in neuroscience, regenerative medicine, infectious diseases, and tumorigenesis, as they provide a tool to study brain health and pathogenesis. In addition, brain organoid technology can be coupled with other technological advancements such as electrophysiology using a patch-clamp technique, optogenetics, genetic engineering, drug screening, and organoid-on-a-chip (Made in ©BioRender—https://www.biorender.com)

circuits, they have become a promising tool to explore the mechanisms of nervous system diseases (Fig. 5.2). Brain organoids have been used to model neurodegenerative diseases such as Alzheimer's disease [20, 21] and Parkinson's disease [9, 14, 22], brain tumorigenesis, Zika virus infection to the brain [12, 23] and neurological COVID-19 [24].

Technical Principles of Brain Organoid Formation

As mentioned above, a hallmark of brain organoids that makes them different from neurospheres is forming cytoarchitectures and tissue morphogenesis of the former [25]. This property allows brain organoids to recapitulate region-specific brain architectures. Following a paradigm of directed differentiation, an original approach toward organoid culture was developed by Yoshiki Sasai to derive cortical

layers from human PSCs using a three-dimensional system termed the serum-free culture of embryoid body-like quick-aggregation (SFEBq) [26]. Since then, various approaches have been devised to generate brain organoids from PSCs. Fundamentally, we will summarise four technical principles employed for the derivation of brain organoids, including (1) factor-primed, (2) self-patterned, (3) fusion, and (4) co-culture approaches (Fig. 5.3).

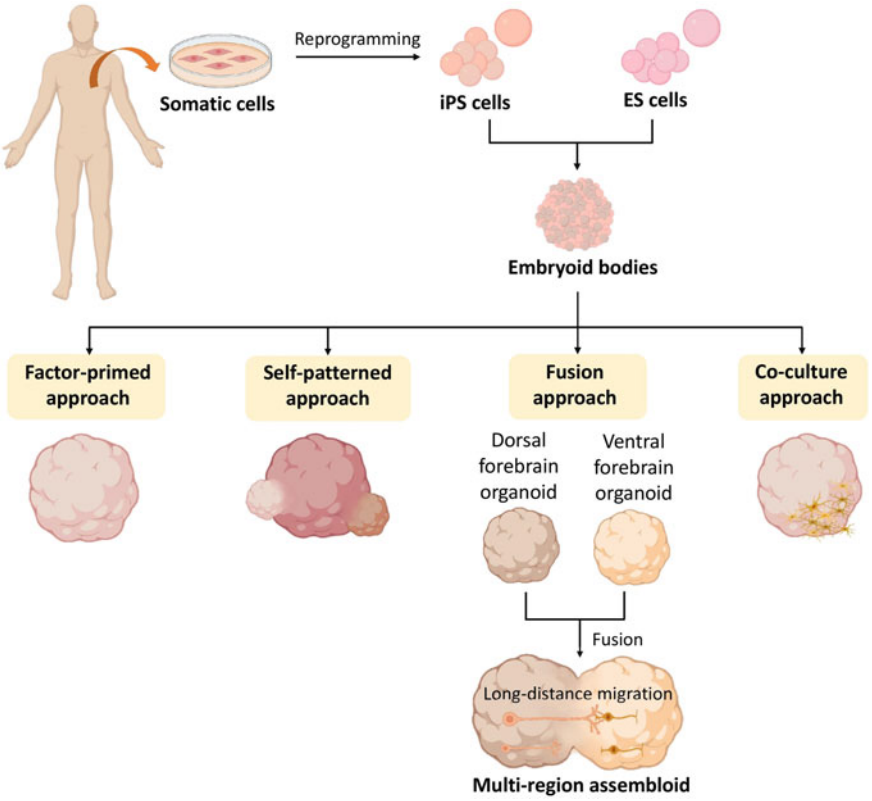

Fig. 5.3 Brain organoids can be derived from human iPSCs and ESCs through the aggregation of EBs using four different methods. The factor-primed approach offers a consistent and reproducible method. The self-patterned approach can deliver diverse cell heterogeneity and extensive morphogenesis. The fusion approach is suitable for the derivation of at least two distinct interconnected yet defined compartments, for example, dorsal and ventral forebrain regions. The co-culture approach gives rise to a brain organoid harbouring not practical cells derived from neural differentiation such as microglia or brain tumour cells (Made in ©BioRender—https://www.biorender.com)

Factor-Primed Approach

Brain organoids can be derived by defined factors. To this end, a factor-primed approach, by which defined extrinsic and trophic factors are added into the culture medium, can be adopted to prime human PSCs to differentiate along neuroectodermal lineages. Scaffolds can also be included in the system to instruct cytoarchitectures and morphogenesis. A well-established protocol of the factor-primed approach is serum-free culture of embryoid bodies (SFEBq), which has been utilised to generate forebrain [27–29], midbrain [9], cerebral cortex [30], cerebellum [31, 32], hippocampus [33], neocortex [34] and pituitary [16]. Moreover, this approach has led to a recapitulation of rostral-caudal organogenesis [35]. A key advantage of using the factor-primed approach is relatively more consistent in cellular heterogeneity and a higher degree of differentiation than the self-patterned approach (see below). However, less advanced-stage morphogenesis is a drawback of this approach as opposed to the other methods.

To avoid limited morphogenesis, step-wise protocols for priming PSCs and their progenies with guiding factors have been established, in which a transient induction by extrinsic and trophic factors is employed to derive radial organisation of the cerebral cortex midbrain organoids and hypothalamic organoids [12, 36]. This temporal manipulation of cell signalling allows brain organoids to be further self-instructed upon removing or diluting the signals. Moreover, the biomaterial poly(lactide-co-glycolide) copolymer (PLGA) can be successfully applied for priming cell attachment and hence facilitating morphogenesis of the organoids around the scaffolds [36]. One study has compared PLGA with carbon fibres for the generation of midbrain organoids and found an increase in expression levels of genes specific to dopaminergic neurons from carbon fibre-primed cultures, structurally more stable than PLGA and does not alter the pH of culture environments [37]. In addition, micropatterned arrays made from the organosilicon polydimethylsiloxane have been shown to improve the derivation of forebrain organoids with homogeneous and singular neural rosettes [38].

Self-Patterned Approach

In contrast to the aforementioned factor-primed approaches, self-patterned approaches are organoid derivation techniques utilising the ability of spontaneous differentiation and subsequently spontaneous morphogenesis of PSC aggregates [39, 40]. Hans Clevers pioneered this protocol for the development of intestinal organoids [41]. Later on, cerebral organoids were successfully derived from matrigel-embedded embryoid bodies. A key success of this method came from using a spinning bioreactor to enhance absorption of nutrients and trophic factors and allow the aggregates to develop self-patterned morphogenesis in a free-floating format [8]. Importantly,

this technique led to a generation of various cell lineages belonging to the forebrain, midbrain and hindbrain in single organoids, indicating a potential of the self-patterned approach to model diseases of the human brain, which might require a crosstalk mechanism among different brain regions.

Nonetheless, in contrast to the signal-primed approach, two drawbacks of this method are a massive cell death inside the organoids and an inconsistency of cellular heterogeneity in the organoids. To increase nutrient absorption and oxygen diffusion and reduce cell death, a multi-well spinning bioreactor system has been engineered for simultaneous expansion of brain organoids, improving the growth of brain organoids and increasing the efficiency of organoid derivation [12]. Furthermore, to overcome a limited expansion of self-patterned brain organoids, genetic deletion of phosphatase and tensin homolog (*PTEN*) led to enhanced cell proliferation of ventricular and outer neural progenitors, in agreement with expansion and folding of human cortical organoids [42]. Moreover, Ming and colleagues have recently developed the self-patterned approach by combining the sliced neocortical organoid (SNO) technique to increase the diffusion of nutrients and trophic factors into SNO, leading to higher cell viability and more expansion of the organoids [43].

Fusion Approach

For factor-primed approach and self-patterned approaches, key drawbacks are limited heterogeneity and uncontrolled cellular diversity, respectively. The latter also usually comes with the irreproducibility of tissue morphogenesis. Specifically, an uncontrolled size of brain regions and tissue organisation are hurdles of this approach. Hence, a novel method has been devised to improve brain organoids' quality in heterogeneity and reproducibility. This is a fusion approach whereby different regions of brain organoids can be fused from individual region-specific brain organoids. Organoids of different brain regions can be fused to generate an expanded architecture, so-called "assembloids". This approach connects multiple brain regions in vitro for long-range and multi-synaptic interconnection. The fusion approach has been used to study the migration of human GABAergic interneurons and to integrate cortical circuits between neurons from the ventral to the dorsal forebrain [44]. This circuit integration comes from interneurons and glutamatergic neurons, which can be found in a microphysiological niche. The authors also utilised the model to study Timothy syndrome and observed a defective neuronal migration. The migration of GABAergic interneurons from ventral to dorsal forebrain is CXCR4-dependent [45]. A similar study has modelled the development of human medial ganglionic eminence of the ventral brain, which hosts neurogenesis of cortical interneurons. Two different region-specific organoids, medial ganglionic eminence organoids and cortical organoids, were fused to observe the migration and integration of interneurons produced by the former [46]. In addition, a method aiming at the generation of thalamus-cortex assembloids by fusing thalamus-like brain organoids to cortical organoids has also been established. Remarkably, the reciprocal thalamocortical

projections between the thalamus and cortex were observed in the fused assembloids [13]. Using the fusion approach, a human multi-synaptic circuit has been recently demonstrated by generating the cerebral cortex or the hindbrain/spinal cord assembled with human skeletal muscle spheroids to generate 3D cortico-motor assembloids [47]. A novel method has been invented for which the midbrain-to-forebrain mesocortical pathway was modelled. This method utilises a hexagonal acoustofluidic device to generate dynamic acoustic fields that can move and fuse one organoid with another in a contact- and label-free manner [48]. Nonetheless, even though the fusion approach offers a path to engineer and expand multi-regional brain organoids with high reproducibility, not all aspects of brain physiology can be implemented, for example, brain-microglia interaction and blood-brain barrier.

Co-culture Approach

A common key limitation in factor-primed, self-patterned, and fusion approaches is that not all cell types present in brain tissues can be obtained from these methods, especially cells belonging to other germ layers such as microglia and endothelial cells. Thus, co-culture protocols have been developed to obtain a complete niche or systems by adding particular cell types into or onto brain organoids.

In order to incorporate microglia into brain organoids, microglia were differentiated from human iPSCs and were tested for their interaction with brain cortical organoids lacking microglia. Upon addition of microglia, by day 3, the cells had migrated into the organoids. The formation of activated microglial clusters was observed when the injury was applied to the organoids [49]. A similar study investigated the role of microglial co-culture in Alzheimer's pathology using brain organoids with Aβ aggregation. The authors found that integrating microglial co-culture can attenuate the accumulation of Aβ plaques [50]. Microglia migrated faster into dorsal organoids than ventral organoids in a comparative study between dorsal and ventral organoids. Immune response upon microglial incorporation was also altered. Specifically, microglia-incorporated dorsal organoids possess higher anti-inflammatory cytokine secretion than ventral organoids, whereas microglia-incorporated ventral organoids express higher TNF-α upon treatment of Aβ42 oligomers [51]. Further, to closely mimic brain microenvironments, Gage and colleagues have successfully transplanted cerebral organoids into adult mouse brains and have established that the engraftment can lead to extensive neuronal differentiation and maturation, gliogenesis, axonal outgrowth, integration of microglia, and vascularisation of endothelial cells [52].

The co-culture approach can benefit from studying the normal physiology and neurological diseases and elucidating tumorigenesis. In one study, cerebral organoids were formed to model gliomagenesis [53]. The cancer cells can infiltrate into and proliferate in the organoids after co-culturing with patient-derived glioma stem cells. Moreover, gap junction mediated-interconnecting microtubes can be observed using two-photon microscopy, facilitating the tumour invasion. In addition, co-culture

approaches can offer a means to serially expand brain tumours into subsequent organoids [63] and understand tumour heterogeneity [54].

With all these four techniques for derivation brain organoids, including factor-primed, self-patterned, fusion, and co-culture approaches, fruitful information has been made regarding fundamental neuroscience, developmental biology, tumorigenesis and drug discovery. Hypotheses for specific research purposes will guide which technique should be employed for the generation of brain organoids. Future approaches may combine several of these techniques to better recapitulate the brain's anatomy and physiology.

Brain Organoid and Neurodegeneration

Neurodegenerative diseases, including Alzheimer's diseases (AD), Parkinson's diseases (PD), Amyotrophic lateral sclerosis (ALS), and Huntington's disease, are prevalent in the elderly worldwide. Previously, studies with human brain tissue, cell cultures, and animal models have been used to study the mechanisms of diseases. Human cerebral organoids and several 3D culture systems exhibit key neuropathological features of the diseases and can be used as disease models.

Alzheimer's Disease

Alzheimer's disease is the most common age-related, irreversible, and progressive disease that slowly destroys the brain. Individuals with early AD develop brain grey matter volume loss in many brain regions such as the hippocampus and the basal forebrain. The disease is clinically characterised by cognitive decline, severe memory impairment, and severe enough life-altering. AD. is characterised by the presence of extracellular amyloid beta-protein deposition, so-called amyloid plaque, and intracellular neurofibrillary tangles. Familial AD. (FAD) is caused by variants in the amyloid precursor protein (APP), presenilin-1 (PSEN1), or presenilin-2 (PSEN2). Sequential cleavage of APP by β and γ-secretase results in a production of Aβ peptide, which aggregates into insoluble amyloid plaques. The deposition of amyloid-beta and hyperphosphorylation of tau could be observed in a 3D culture system of human neural stem cells with amyloid precursor protein (APP) and presenilin1 (PSEN1) mutation [20]. Moreover, brain organoids derived from multiple FAD patients induced pluripotent stem cells to develop continuous amyloid deposition and tau hyperphosphorylation in an age-dependent manner [21].

Recently, Cairns and colleagues described a new model of AD. Using HSV-1 infection to a 3D brain model. This model can develop amyloid plaque-like formations, gliosis, neuroinflammation, and decreased functionality [55].

Parkinson's Disease

Parkinson's disease (PD) is the second most common neurogenerative disease after AD. PD is characterised by resting tremor, bradykinesia, rigidity, and postural balance instability. The major cause of clinical symptoms is the degeneration of midbrain dopaminergic neurons. To model PD in brain organoids, midbrain-specific organoids were developed [9, 14]. The midbrain-specific organoids contained functional tyrosine hydroxylase-positive midbrain dopamine neurons (mDAns) after 2 months in culture. These mDAns express midbrain markers, such as FOXA2 or dopamine transporter, and show cytoplasmic neuromelanin accumulation. Patient-specific iPS cells from PD patients could be used to model PD with midbrain-specific organoids. The early reports of PD modelling in midbrain-specific organoids focused on the effects of the LRRK2-G2019S variants. CRISPR-Cas9 has been used to introduce the mutation in control human pluripotent stem cell lines [56] or create isogenic mutation corrected lines from patient-specific cells [22]. Kim and colleagues observed no difference in size between LRRK2-G2019S midbrain-specific organoids compared to control. However, less neurite length of mDAns and lower expression of dopaminergic neuron marker were noted [56]. On the other hand, a smaller number of mDAns and lower complexity of their neurites were observed in the midbrain-specific organoid derived from LRRK2-G2019S mutated patient iPS [22]. Midbrain organoids may also be used to study sporadic forms of PD by exposing the organoids to exogenous stressors, such as MPTP.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a devastating neurogenerative disorder caused by the loss of motor neurons. The most common cause of familial ALS is superoxide dismutase type-1 (SOD1) mutations, resulting in increasing aggregated and soluble misfolded forms of SOD1, leading to the death of motor neurons [57]. Seminary and colleagues generated motor neuron cultures from human iPSC lines carrying mutations in SOD1. Accumulation of insoluble SOD1 can be observed in ALS iPSC-derived motor neurons. However, the heat shock response or stress granule formation in response to protein accumulation cannot be observed [58]. To date, there is no publication using organoids to model ALS. This might be because the motor neurons can be divided into upper motor neurons and lower motor neurons. The upper motor neurons are in the motor cortex, and the lower motor neurons are in the ventral horn of the spinal cord. Therefore, brain organoids cannot mimic the lower motor neurons physiology and environment. Kawada and colleagues developed a protocol to generate a motor nerve organoid from human pluripotent stem cells using a microdevice equipped with a narrow channel to provide a microenvironment for axonal growth. The generated motor nerve organoid mimics the development and dysfunction of a human motor nerve [59]. Later, a protocol to generate a 3D

spinal cord organoid from human induced pluripotent was established [60]. Different spinal cell types were observed with this protocol in the spinal cord organoids and patterned along the rostro-caudal axis, mimicking the ventral spinal cord. Fusing the motor cortex brain organoid to the motor nerve organoid or spinal organoid could be a possible model for further ALS study.

Other Applications

Besides, brain organoids and assembloids could serve as an innovative tool to model pathology and study disease mechanisms from a healthy individual and patient nervous system. Brain organoids and assembloids can be combined with many recent technologies such as optogenetics to use light to control neurons, CRISPR/cas9 for genome editing, patch-clamp for electrophysiology study, and on-a-chip system to control continuous perfused cultures to create more precise models of brain development and diseases.

Brain Organoid for Drug Development and Personalised Medicine

For clinical translation, brain organoids can be used to model patient-specific molecular and cellular pathogenesis, thus guiding the most effective treatment for individual patients, a process called personalised medicine. Personalised organoids can be derived from a specific patient. Briefly, the cells would be obtained from the patient, reprogrammed into iPS cells, and grown brain organoids on a large scale. Personalised brain organoids can be used to test the effectiveness of a compound library (new drug development) to find the ones most appropriate for the patient. Recently, Park and colleagues used 1300 cerebral organoids, including CRISPR/Cas9-edited isogenic lines, from 11 AD patients to assess blood-brain barrier-permeable FDA-approved drugs and purposed a strategy for precision medicine by integrating those cerebral organoids and mathematical modelling. Their results demonstrated the possibilities of drug repositioning and simplified the drug approval process in preparation for precision medicine [61]. In addition, since autism spectrum disorder is a polygenic disease, it is difficult to precisely develop a curable treatment for the patients. To overcome this multi-genetic barrier, cerebral organoids made from the patients via iPS reprogramming have been proposed for personalised drug discovery [62]. However, the production scale of the cerebral organoids is a challenge for the high-throughput drug screening. Specifically, most of the established protocols have been developed using 96-well plates. Therefore, the automation system is required to produce cerebral organoids on a large scale, which will eventually accelerate the development of novel personalised therapeutic strategies for brain disorders.

Conclusion

Brain organoid technology is a powerful tool for researchers to study early human brain development and diseases. Four approaches can be considered for generating brain organoids: (1) factor-primed; (2) self-patterned; (3) fusion; (4) co-culture approaches. Pathogenesis of Alzheimer's, Parkinson's, and ALS diseases, among others, have been successfully modelled using brain organoids. When coupled with genome editing tools such as CRISPR/Cas9, patient-specific brain organoids are key for personalised and precision medicine.

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Chapter 6
Organoids in the Human Reproductive System

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Abstract **Introduction**: The limitation of using the animal as the model for studying human reproductive diseases is that the biological background of the animal may not recapitulate the pathology of human diseases. Therefore, human cell culture technology is progressively developed to replace the use of animal models. Adherent cell culture or two-dimension (2D) culture condition has long been widely applied for a culture of several primary or pathological reproductive cell lines, including Hela cells, an immortal endometrial cell line. However, the data obtained from 2D culture conditions may not always be translated or recapitulated in vivo, especially in the disease mechanisms. Here we review the organoid technology currently applied for the female and male reproductive system, focusing on the progress of technologies used in different reproductive organs and future applications, especially for regenerative medicine. **Methods**: In this chapter, MEDLINE/Pubmed and Scopus databases were used for a survey of the literature. The keywords used for searching were; 3D culture, organoids, male reproductive organs, female reproductive organs, disease modelling, stem cells, personalised medicine. **Results**: Reproductive organoids can be generated from adult stem cells, which are established directly from the healthy or pathogenic tissue of reproductive organs, or pluripotent stem cells, which latter are being differentiated into the reproductive cells. Specific 3D-culture conditions such as extracellular matrices, signalling pathways, or cross-talked between different cell types in the organoid have to be manipulated to successfully maintain the reproductive organoids in the in vitro system. Moreover, the patient-derived reproductive organoids are feasible for developing drug screening tests for individual cancer patients. **Conclusions**: Interestingly, patient-derived reproductive cancer organoids are currently developed for biobanking. The advantages of applying the organoids in modelling human infectious diseases, genetic disorders, and cancers that cause reproductive organ problems. The organoid biobanking will further assist drug development platforms, personalised medicine.

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Keywords Reproductive organs · Pluripotent stem cells · Disease modelling · In vitro culture

Abbreviations

2D	Two-dimension
3D	Three-dimension
3-LGS	Three-Layer Gradient System
AKT	Protein kinase B
ALDH	Aldehyde dehydrogenases
ARM4	Armadillo repeat containing 4
ASCs	Adult stem cells
bFGF	Basic fibroblast growth factor
BMP4	Bone morphogenetic protein 4
BPH	Benign prostatic hyperplasia
cAMP	Cyclic adenosine monophosphate
cCCC	Cervical clear cancer cell carcinoma
CD	Cluster of differentiation
CHD1	Chromodomain Helicase DNA Binding Protein 1
CHIR99021	GSK3 inhibitor
COL4	Collagen type IV
CTNNB1	Catenin Beta 1
CZ	Central zone
DMEM	Dulbecco's Modified Eagle Medium
DNAI1	Dynein Axonemal Intermediate Chain 1
EBs	Embryoid bodies
ECAD	E-cadherin
ECM	Extracellular matrices
EGF	Epidermal growth factor
ERG	Erythroblast transformation-specific (ETS) related gene
EZH2	Enhancer of zeste 2
FBS	Fetal bovine serum
FGF2	Fibroblast growth factor 2
FGF10	Fibroblast growth factor 10
FOXJ1	Forkhead Box J1
FT	Fallopian tube
FTEC	Fallopian tube epithelial cells
FTMSC	Fallopian tube mesenchymal stromal cells
GSK3B	Glycogen Synthase Kinase 3 Beta
hESCs	Human embryonic stem cells
HGF	Hepatocyte growth factor
HGSCs	High-grade serous carcinoma
hiPSCs	Human induced pluripotent stem cells

HLA	Human leukocyte antigen
HOX	Human Homeobox
hPSCs	Human pluripotent stem cells
HPV	Herpes virus
HUVEC	Human umbilical endothelial vein
ICM	Inner cell mass
IFIT1	Interferon Induced Protein With Tetratricopeptide Repeats 1
IFN- β	Interferon beta
IGF1	Insulin-like growth factor 1
IL-6	Interleukin-6
KRT5	Keratin 5
KRT7	Keratin 7
LC	Langerhans cells
LGR5	Leucine-rich repeat-containing G-protein coupled receptor 5
LRRC6	Leucine-Rich Repeat-Containing Protein 6
MEHP	Mono (2-ethylhexyl) phthalate
MUC1	Mucin 1
OSE	Ovarian surface epithelium
PC	Prostate cancer
PCOS	Polycystic ovarian syndrome
PDGF-BB	Platelet-derived growth factor two B subunits
PDXs	Patients-derived xenograft
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinases
PID	Pelvic inflammatory disease
POI	Premature ovarian insufficiency
PZ	Peripheral zone
ROCK	Rho kinase
ROR1	Receptor Tyrosine Kinase Like Orphan Receptor 1
RSPO1	R-Spondin 1
SCJ	Squamocolumnar junction
SCT	Syncytiotrophoblast
SOX17	SRY-Box Transcription Factor 17
SPINK1	Serine Peptidase Inhibitor Kazal Type 1
SPOP	Speckle Type BTB/POZ Protein
SSEA3	Stage-specific mouse embryonic antigen 3
SSEA4	Stage-specific mouse embryonic antigen 4
STAT3	Signal transducer and activator of transcription 3
TE	Trophectoderm
TGFβ	Transforming growth factor β
TMPRSS2	Transmembrane Serine Protease 2
TP63	Tumor Protein P63
TZ	Transformation zone (Cervical organoid)

TZ	Transitional zone (Prostate organoids)
WNT	Wingless-related integration site
Y-27632	ROCK inhibitor

Introduction

The human reproductive system consists of reproductive organs and related structures. Both female and male reproductive systems generally have primary and secondary organs or structures based on their rules. The primary reproductive organs can be called "gonads", which are the ovary in females or testis in males. The major functions of the gonad are producing the gametes, oocyte or sperm and hormones. While the secondary reproductive organs such as the fallopian tube or uterus in females and epididymis or prostate gland in a male are responsible for the maturation of the gametes as well as the growth of the embryo [1], the in vitro culture of the cells derived from the reproductive organs is widely applied to understand cell morphology, physiology, and the mechanisms underlying specific diseases of human reproductive organs. To date, the researchers have developed several effective protocols for culturing cells or tissue from different reproductive organs. The in vitro culture can be conventionally carried out under adherent conditions, whereas the cell attaches to the surface of the treated culture dish so-called two-dimension (2D) culture. The 2D culture of primary or tumour cells derived from the reproductive organs demonstrated numerous insights into the physiology and biology of their organ origins. Although 2D culture conditions represent many advantages, such as being easy to manipulate, the cell structures can be clearly defined under the microscope. However, the 2D culture condition is proved that this condition is non-physiological and, therefore, does not represent a complex microenvironment of reproductive tissues or organs [2, 3]. Reproductive cells that grow under the 2D conditions lack body microenvironments, including cell-cell interaction and extracellular matrices (ECM). Most primary or tumour cells derived from reproductive organs display a homogenous phenotype.

Alternatively, three-dimensional (3D) culture is a culture condition that allows the cells to grow and communicate with the neighbouring cells or surrounding extracellular matrices networks. Moreover, the genetic alteration and rapid loss of their phenotype after prolonged culture under 2D condition make them unsuitable for recapitulating the microenvironment of the in vivo condition. Therefore, the data obtained from 2D conditions may be inadequate for being translated to the function of reproductive organs [2, 3]. An organoid is one of the most advanced 3D culture conditions. Therefore, this chapter summarises the recent advance of organoids in female and male reproductive organs, including the principle of cell interaction, signalling pathways that mimic the organoids, type of organoid of reproductive organs, and the disease modelling of reproductive organoids.

Cell Sources for Derivation of Reproductive Organoids

Typically, human reproductive organoids are derived from the adult stem cells (ADCs), such as human ovarian epithelial cells isolated from follicular fluid (Fig. 6.1a) or human pluripotent stem cells (hPSCs; Fig. 6.1b). Due to the differences in the cellular and molecular biology of the ADCs and PSCs, care must be considered to develop derivation and culture protocols. For instance, ADC-derived reproductive organoids contain tissue-specific stem cells, losing their stemness after prolonged culture can occur. Therefore, manipulating the culture medium by supplementation with tissue-specific cocktails of cytokines or growth factors can maintain their stem cell niche and physiological environment [4]. On the other hand, more complexities of differentiation PSCs into specific reproductive cells due to the differentiation processes involve several signalling pathways, transcription factors or extracellular matrices [5]. Thus far, using PSCs as the starting cell type could hurdle the successfulness of reproductive organoid derivation. However, one of the most challenging of using PSCs to derive the reproductive organoids is that cells from patient-specific diseases can be harvested and reprogrammed to human PSCs and the subsequent generation of the specific disease organoids. The hiPSC, together with organoid technologies, support the simulation and investigation of pathological diseases in vitro. Besides, the advancement of CRISPR/Cas9, a powerful gene-editing technology, allows researchers to precisely create or repair specific gene mutations. Furthermore, applying iPSC and gene editing technologies such as correcting gene mutations that cause the disease can achieve a more effective therapy [6].

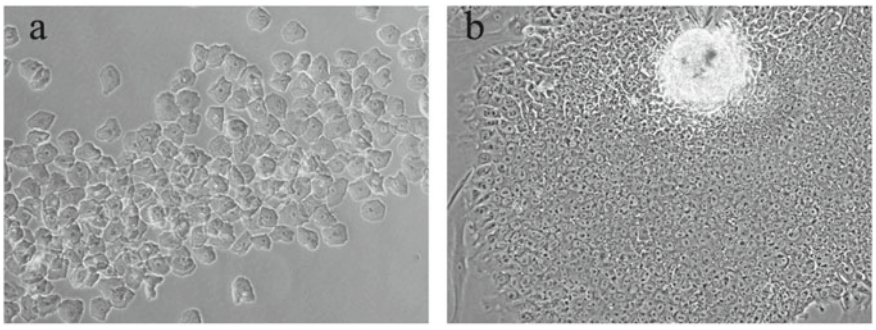

Fig. 6.1 Cell sources for generation of human reproductive organoids. The reproductive organoids can be established using somatic cells isolated from the reproductive organs or reprogramming of somatic cells to human pluripotent stem cells (hPSCs). For instance, ovarian epithelial cells obtained from human ovarian follicular fluids during ultrasound-guided oocyte retrieval (**a**) and hPSCs generated from reprogramming of dermal skin fibroblast cells (**b**). Magnification 100 X

Organoids as a Model for Female Reproductive Diseases

The researchers have demonstrated the major advantages of using the reproductive organoid as a disease model, including displaying the pathological processes of some diseases such as ovarian cancers or endometriosis [7, 8]. The disease-specific organoid models assist the understanding of how the diseases develop and progress. Moreover, the organoid disease models are very useful for developing potential diagnoses and treatments.

Ovarian Organoids

The ovaries are the gonadal organs that are located near the uterus, in the abdomen. The ovary produces the female gamete, oocyte and hormones such as estrogen, progesterone and inhibin [9]. The physiology of the ovary is complex due to its works closely under the control of pituitary–gonadal and ovarian hormones. Anatomically, the ovary comprises the outer layer of epithelial cells, which suggests that it is related to neogenesis and cancer [10]. The ovarian surface epithelium (OSE) superficially covers the dense connective tissue, tunica albuginea which is sometimes related to polycystic ovarian syndrome (PCOS), a syndrome that involves the difficulty of the mature follicle to ovulate [11]. Two major portions of the ovary are the cortex and medulla. The ovarian cortex is composed of a framework of ovarian follicles and stromal cells. The oocyte develops inside the follicles under the closed control by the hormones. The growth of the follicles is dynamic and complex. The differentiation of stromal cells into theca interna and theca externa plays an important role in follicular development. Therefore, the cells' complexity and dynamic inside the ovary must be understood before generating ovarian organoids. Due to the ovarian cancers primarily emerging from the OSE, much research focuses on deriving ovarian cancer organoids using the ovarian surface epithelium derived from cancer patients. Physiologically, OSE is closely related to ovulation and transformation of the normal epithelial cells and cancer progression. In the normal condition, after the ovulation, the repairing process of OSE begins with proliferation, migration, and finally regeneration of the ovarian surface by coordinating the secretion of the new extracellular matrices and proteolytic enzymes [12]. These dynamic processes can be explored through organoid technology using 3D culture and plating the organoid onto a combination of extracellular matrices, collagen, and OSE-derived extracellular matrix [12]. Ovarian cancer is one of the highest fatality rate cancers in females [13]. Ovarian cancer organoids have been proved to be a very useful model in pre-clinical research. The researchers could discover the cancer mechanisms such as progression and recurrent cancer and resistance to the chemotherapy. Therefore, the new diagnostic, therapeutic or preventive platforms using ovarian organoids are progressively improved. Technically, to derive the ovarian cancer organoids, the ovarian tumour biopsies must be dissociated into fragments, embedded in a scaffold and cultured in a cocktail of culture

medium containing signalling factors, cytokines, and growth factors to be optimised for only ovarian cell type. Using the culture medium of the other types of cancer organoid for culturing the ovarian cancer organoid may limit the growth and development of ovarian cancer organoids [14]. Maenhoudt et al. [14] demonstrated the low efficiency of ovarian organoid formation after cultured in the culture medium for endometrial and endometrium cancer. Moreover, the concentration of growth factors such as basic fibroblast growth factor (bFGF), FGF10, transforming growth factor β (TGFβ) pathway inhibitor A83-01, addition of hepatocyte growth factor (HGF) or insulin-like growth factor 1 (IGF1), relatively influenced the stage of ovarian organoid development.

Despite using tumour biopsies as the starting material, the OSE can be surgically scratched with the cytological brush and cultured in the medium supplemented with fetal bovine serum (FBS) under 2D culture conditions [15]. Subsequently, 3D condition using Matrigel-coated culture dish, together with the culture medium containing 2% Matrigel, can be applied to grow OSE organoids. The presence of laminin, fibronectin, and gelatin in Matrigel provides a suitable extracellular matrix for the OSE to form the 3D structure, presenting a single epithelial lining with the hollow lumen within 48 h [15]. Besides, the OSE organoids can develop and grow according to the duration of in vitro culture. E-cadherin (ECAD) expression, which involves cell-cell junction, can be detected within a few days after forming the organoid structure.

In comparison, collagen type IV (COL4) expression, a basement membrane component, was detected at the basal membrane around one week onward after forming the organoid [15]. The ovarian cancer organoid recapitulates the pathophysiology of cancer. Therefore, optimising the culture medium for ovarian cancer organoids is important for achieving their most effective growth and development. It is generally accepted that the cancer organoid models have a strong potential for in vitro and pre-clinical research. The pre-clinical results obtained from ovarian cancer organoids provide personalised therapeutic options and assist in clinical decision making [2].

Besides cancer models, up to date, there is a lack of reports about generating the ovarian organoid from other diseases, for instance, polycystic ovarian syndrome (PCOS) or premature ovarian insufficiency (POI), which is associated with not only the number of mature oocytes but also metabolic and endocrine complications [16]. Having the POI or PCOS organoids would support and provide information regarding the disease mechanism and the development of the new treatment.

Fallopian Tube Organoid

The fallopian tube (FT) is the bilateral organ that connects the ovaries to the uterus. The fallopian tubes play an important role in the female reproductive system, including maturation of the oocyte, gametes and embryo transportation, fertilisation, and embryo development [17]. Fallopian tubes can be divided into three

parts: infundibulum, ampulla, and isthmus [18]. The epithelium of the fallopian tube contains ciliated and secretory cells. After ovulation, the oocyte is released and captured by the cilia of the fallopian tube. The oocyte is guided into the ampullary-isthmus junction, where fertilisation occurs. Later, the fertilised zygote would travel to the uterus through the movement of the cilia. The secretory cells secrets some essential secretion to support the motility of the oocyte or zygote [18]. Anatomical or functional abnormalities of the fallopian tube cause several clinical diseases, for example, high-grade serous ovarian cancer [19] or infertility [20]. The major limitations of accessing the FT are technically invasive, and continuing long-term follow-up is almost impossible. Hence, the FT organoid may provide ease and recapitulates the in vivo environment of the FT. In 2015, Eddie and colleagues [21] reported the success of the generation of 3D human fallopian fimbriae using an alginate matrix to support the growth of human fallopian fimbriae ex vivo.

Subsequently, Chang et al. [22] isolated the primary fallopian tube epithelial cells (FTEC), cultured under 2D culture condition and followed by suspension 3D culture condition. These conditions allow the cells to multiply while maintaining their stemness, as confirmed by the expression of normal stem cell markers, including LGR5, SSEA3 and SSEA4. Moreover, FTEC expresses CD24, CD44, CD117, ROR1, CD133 and ALDH—cancer stem cell markers. Later, the authors generated the organoid by mixing FTEC with fallopian tube mesenchymal stromal cells (FTMSC) and human umbilical endothelial vein (HUVEC) and cultured under 3D condition. Their culture medium consists of DMEM supplemented with Wnt3a, RSPO1, FGF10, EGF, noggin, a ROCK inhibitor, nicotinamide, and TGF-β R kinase inhibitor IV the presence of Matrigel for 21 days. Both ciliated and secretory cells in the 3D human fallopian fimbriae maintain their normal architecture for up to 7 days, demonstrating the feasibility of generating FT organoids. The established FT organoids in this study consisted of two important cell types of the fallopian tube, secretory and ciliated cells.

Despite the generation of the FT organoid from the primary cells, human iPSCs can be used as the starting cells, as reported by Yucer et al. [23]. The process of differentiation of human iPSCs to fallopian tube epithelial cells is very complex. Human iPSCs have to be first induced to mesodermal lineage, followed by the formation of the Mullerian duct, as the reproductive system is closely related to the urinary system [24]. Yucer et al. [23] demonstrated cell fate decisions during differentiation of iPSCs to fallopian tube epithelial cells controlled by the proteins and growth factors. Mesoderm development can be induced by Activin A and CHIR99021, regulating the intermediate mesoderm fate by stepwise addition of BMP4, followed by Wnt4 and follistatin to replicate the Mullerian duct. Therefore, the addition of cytokines or growth factors in the culture medium in each step and determining the localisation and expression of the FT markers are extremely important. Besides the cytokines or growth factors, a scaffold such as Matrigel is necessary for FT development. Thus far, Yucer et al. [23] demonstrated that the iPSC-derived FT organoid exhibits the anatomy and physiology of human FT as confirmed by estrogen and progesterone responses.

Moreover, the cilia and secretory cells and the folding epithelium are identified in the FT organoid. At the molecular level, it has been found that the stem cell in the FT organoid maintains its stem cell through the activation of WNT and NOTCH signalling. Inhibition of NOTCH signalling leads to upregulation of ciliogenesis genes such as *ARMC4, DNAI1, FOXJ1*, and *LRRC6,* increasing ciliate cells in the FT organoid [25].

The diseases of FT are generally the infections that cause damage and scar formation of the tubes, for instance, pelvic inflammatory disease (PID), which is primarily caused by bacterial infection. Understanding the disease's initiation and progression is necessary to develop the new diagnostic and therapeutic tools for effective treatment outcomes. One promising FT organoid-disease model is high-grade serous carcinoma (HGSCs), which mostly arise from the secretory cells of the FTE [26, 27]. As mentioned earlier, Notch signalling involves initiation, progression and metastasis of HGSCs [25]. Importantly, the differentiation of the secretory cells of the FTE can be blocked and controlled by adding a Notch γ-secretase inhibitor into the FT organoid culture condition. Therefore, the information regarding controlling NOTCH signalling can be further investigated by using HGSCs fallopian organoid. This may provide a novel and effective treatment for HGSC patients [28].

Endometrial Organoids

The human uterus consists of three layers that lining from the luminal to the serosal parts, including endometrium, myometrium, and perimetrium. The endometrium is a highly dynamic tissue that cyclically responds to the steroid hormones and proteins for menstruation and embryo implantation [29]. Many gynecologic diseases are involved with the endometrium, including endometriosis, dysmenorrhea, infertility and cancer. Among these, endometrial cancer is commonly found in the female reproductive system [30].

Endometrial organoids can be derived from normal endometrial or pathogenic endometrial tissue [8]. The primary endometrial cells were first dissociated and then embedded in the Matrigel droplet [8, 31]. Unlike the ovarian organoid, Turco et al. [8] demonstrated that the composition of the culture medium of the endometrial organoid could be the same as the one commonly used for culture other organoids. Like mouse endometrial organoid [31], human organoid exhibits glandular organisation, apicobasal polarity, mucus production, and response to sex hormones [8, 31]. Thus far, the established endometrial organoids have recapitulated the anatomy and physiology of *the* in vivo endometrium. Despite the generation of endometrial organoids from the endometrial tissue, recently, for the first time, human iPSC-derived endometrial organoids can be successfully established under the stepwise protocol [32]. The differentiation processes involve inducing human iPSC to form the embryoid bodies (EBs), followed by culturing and treating the EBs with the sequential cocktails of cytokines and growth factors including, CHIR99021, a GSK3B inhibitor/CTNNB1 pathway agonist, fibroblast growth factor 2 and 9, retinoic acid,

NOGGIN, 17b-estradiol, and PDGF-BB for over 14 days, resulting in the primitive streak, coelomic epithelium and finally endometrial stromal fibroblasts, respectively. Furthermore, decidualisation of the human iPSC-derived endometrial stromal fibroblasts was confirmed by treatment with estradiol, medroxyprogesterone acetate, and 8-bromoadenosine 3′–5′-cyclic monophosphate [32, 33].

In order to recapitulate the pathophysiology of cancer, Turco et al. [8] derived the endometrial organoid by using the endometrial adenocarcinomas from postmenopausal women and demonstrated that this cancer-derived endometrial organoid has a glandular origin, as confirms by the expression of glandular markers including MUC1 and SOX17. Although several pathogenic characteristics in these endometrial adenocarcinoma organoids were observed, such as pleomorphic cells with hyperchromatic nuclei, disorganised endometrial epithelium, basement membrane detaching and cell invasion, into the Matrigel, they maintain their chromosomal integrity after prolonged culture [8]. Moreover, these patient-derived endometrial organoids can survive and grow after freeze-thaw cycles, making them a valuable tool for the biobank of patient-derived organoids. Patient-organoid biobanking may assist new endometrial cancer treatment strategies.

In order to improve the microenvironment of the endometrial organoid, the endometrial epithelium can be co-cultured with the stromal cells [34–36]. In the normal endometrium, there is a cross-talk between the stromal and endometrial epithelial cells. The stromal cells are responsible for the endometrial epithelium's proliferation, differentiation, and decidualisation [37]. Besides supporting the growth of endometrial epithelium, stromal cells may cause some pathogenic conditions like endometrial carcinoma [34–36] and endometriosis. The recent finding of Esfandiari et al. [38] emphasised the important role of stroma cells in human endometriosis using endometriosis organoids. The results demonstrated the similar methylation alterations patterns of the Human Homeobox (HOX) cluster, A-D and HOX cofactors in ectopic/eutopic endometrium tissues and ectopic/eutopic endometriosis organoids compared with normal endometrium. In addition, the endometriosis organoids in their study maintain epigenetic changes as confirmed by the conserved pattern of methylation alterations in the endometriosis organoids and tissue.

Therefore, the complex organisation of endometrial epithelium and stromal cells provides a model for studying the epithelium-stroma interactions in vivo. Moreover, endometriosis organoids represent a novel disease model to determine the genetic and epigenetic mechanisms that underlie human endometriosis, which can be useful to the development of new therapeutic platforms.

Cervical Organoid

The cervix connects the uterus to the upper part of the vagina. Anatomically, the cervix can be divided into three parts; endocervix, the squamocolumnar junction (SCJ) and ectocervix. There are two different epithelial linings in the cervix, the

columnar epithelium at the endocervix, whereas the squamous epithelium with non-keratinocyte at the ectocervix. Importantly, the transformation zone (TZ), the transition area between the two epithelial lineages, has been found at the SCJ. The SCJ undergoes remodelling in response to the steroid hormones during puberty, pregnancy and menopause during normal physiological conditions [39]. Importantly, the SCJ region of the cervix is widely known to be the area where cervical cancer emerges after the HPV infection to the cervix. Therefore, creating the cervical or SCJ organoids will assist the researchers to better understand the disease mechanism and develop a new and effective treatment.

With the limitation of accessing human cervical tissue, Jackson et al. [40] alternatively developed a human cervical organoid by a culture of the commercial cervical keratinocytes, fibroblast and myeloid cell line followed by maturation and purification into Langerhans cells. The authors focused on LC cells because it is relatively scarce in the cervical mucosa and that HPV infection can be found. Although this human cervical organoid supports a better understanding of the HPV-infection microenvironment, it is not directly derived from the patients. Maru et al. [41] demonstrated that the normal SCJ samples could give rise to the organoid for the first time. Under the suitable 3D culture condition, the organoid exhibits the cuboidal SCJ cells, express SCJ markers and, more importantly, consists of squamous cells resembling transformation zones. Interestingly, the organoid culture condition reported in their study can be prolonged-cultured without any genetic modification.

It is very important to distinguish the markers specifically related to the specific epithelial cells of the endocervix and ectocervix. Endocervical organoids express KRT7, while the ectocervical organoids express KRT5 and TP63, the basal markers. Besides, the in vitro growth of cervical SCJ organoids depends on supplementation of EGF, Noggin, Y-27632, RSPO1, and Jagged-1 in the culture medium [41].

The organoid of a cervical clear cancer cell carcinoma (cCCC), a rare subtype of cervical cancer, has been successfully generated by Maru et al. [41]. cCCC organoids were grown under the double layers of Matrigel. By using genomic analysis, the authors detected mutations in both cCCC organoids and CCC compartments. Interestingly, these cCCC organoids showed sensitivity to anti-cancer drugs, including paclitaxel, cisplatin, and gemcitabine. These results indicated that cCCC organoids might assist the therapeutic finding for cCCC patients.

Trophoblast Organoids

Two types of cells emerge dominantly inside the embryo at the blastocyst stage, the inner cell mass (ICM) and trophectoderm (TE). The ICM differentiates further into the body, whereas the TE gives rise to trophoblasts which play a crucial role in fetal placenta formation.

Trophoblasts are considered as the stem cells that differentiate into villous and extravillous pathways. In the villous pathway, cytotrophoblast cells form multinucleated syncytiotrophoblasts (SCT). In the extravillous pathway, cytotrophoblast cells

acquire an invasive phenotype and differentiate into either (i) interstitial extravillous trophoblasts, which invade the decidua, or (ii) endovascular extravillous trophoblasts, which involves maternal vascularisation [42]. Trophoblast function deficiency causes many complications such as miscarriage, recurrent abortion, preeclampsia and preterm abortion [43, 44].

Immortal human placental cell lines have been isolated and developed from the choriocarcinoma tissue such as BeWo, JEG-3 and JAR [45]. Although these cell lines have several advantages like ease of access, less complication in manipulating their genes, or lack of ethical concerns, the immortal characteristics, especially their malignant transformations, may not represent the in vivo conditions. Genetic modification of primary trophoblast cells by using genes encoded for simian virus 40 large T antigen results in the immortal trophoblast cell lines [46] overcome the limitation of using trophoblast cell lines derived from carcinoma tissue.

Alternatively, trophoblast cells can be differentiated from human embryonic stem cells (hESCs) by treatment with bone morphogenetic protein 4 (BMP4), resulting in morphological and functionally similar to trophoblast cells [47, 48]. In addition, our previous study demonstrated that BMP4 could induce both normal and abnormal karyotypic hESC lines into trophoblast-like cells [49]. Unfortunately, some hESC-derived trophoblast cells differently displayed trophoblast-specific markers, global gene expression profiles, and HLA status to those primary trophoblast populations [50].

The recent reports demonstrate the advancement of basic and clinical research relating to placenta development using trophoblast organoid models. Sheridan et al. [51] successfully establish and differentiate the trophoblast organoid from the first-trimester human placenta within 3 weeks. Interestingly, the trophoblast organoid can be prolonged cultures under their 3D culture condition for more than one year. The optimal composition of the culture medium for growing the trophoblast organoids is very important. Supplementation of cocktails including FGF2, HGF, EGF, and CHIR99021 and R-spondin-1 (WNT activators), Y-27632, PGE2 (cAMP/AKT activator) and a TGFβ inhibitor influenced the success of trophoblast organoid generation [51]. Not only the villous-like organoid containing syncytiotrophoblasts and villous cytotrophoblast can be generated, but also it can be prolonged culture. Besides, applying enzymatic dissociation of trophoblast organoids with Accutase enhances expansion and propagation of the trophoblast organoid [51]. Although there are a few reports regarding disease-specific trophoblast organoids, the mechanism of how the Zika virus impairs the fetal-maternal interface can be explored using the human trophoblast organoid [52].

Organoids as a Model for Male Reproductive Diseases

The male reproductive system consists of the external structures: penis and scrotum, the internal structures: testes, epididymis, ductus deferens and accessory gland including seminal vesicles, prostate gland and Cowper's glands. The major functions of male reproductive organs are producing, maintaining and transporting the sperm. The testis also produces and secret male sex hormones such as testosterone to maintain the male reproductive system.

Testicular Organoid

The testicular microenvironment can be recreated in vitro by testicular organoid technology, allowing several applications such as microanatomy, physiology, drug toxicity test, and disease modelling.

Similar to female reproductive organoids, cell to cell interaction and ECM plays an important role in the testicular organoid generation. Baert et al. [53] demonstrated that the growth of testicular cells on the decellularised adult testicular ECM resulted in spheroidal structures. Although the morphology of these structures does not resemble the human testis, the expression of tight junction proteins in the Sertoli cells and the production of testosterone and inhibin B indicate their organoid characteristics. Alves-Lopes et al. [54] developed a system to generate testicular organoids by applying Three-Layer Gradient System (3-LGS). These three layers consist of a drop of Matrigel on the surface of the culture plate as the first layer. Then, the rat interstitial and tubular fraction combined with Matrigel was placed as the second layer. Finally, the Matrigel covers the two layers. Three-Layer Gradient System allows the cellular reorganisation of testicular organoid and spherical-tubular structures that recapitulate the testicular niche.

Sakib et al. [55] successfully generated human testicular organoids using microwell aggregation. The testicular organoids recapitulate the testicular niche due to the interaction of cells residing in the organoids, including germ cells, Sertoli cells, myoid cells and Leydig cells. Claudin 11 and occluding, the tight junction proteins were expressed by Sertoli cells in the organoids. Moreover, after treatment with mono (2-ethylhexyl) phthalate (MEHP), the increase of germ cell autophagy was determined. These results convinced that testicular organoids could be used for drug screening tests which can be beneficial for the development of personalised medicine.

Testicular infection caused by microorganisms, including viruses, leads to the testicular tissue's inflammation and impairs sperm and hormonal production. It has been shown that Zika virus infection caused declining sperm number and testosterone levels, indicating that Zika virus infection may affect male infertility [56]. To explore the effect and mechanism of Zika virus infection on the testicular function, Strange et al. [57] generated the testicular organoid using a combination of 2D and

3D conditions. Firstly, adult primary LC, SC, peritubular cells and spermatogonia were propagated and cultured under 2D conditions. Later, cells were harvested and allowed to aggregate in the enriched medium containing testis ECM under 3D conditions using ultra-low culture dishes. The established testicular organoid was further infected with the Zika virus, and the results confirm that Zika virus efficiency infects their testicular organoid.

Moreover, Zika virus infection increases the expression of antiviral genes, including IL-6, IFN- β and IFIT1 [57]. Currently, data demonstrated that SARS-CoV-2, which causes COVID-19, can invade and damage the human testis as the viral particles can be detected in the semen and testicular tissue of the COVID-19 infected patients [58, 59]. Therefore, testicular organoid can be applied to study both short- and long-term implications of SARS-CoV-2 infection.

Prostate Organoids

The prostate is the largest male accessory gland surrounding the proximal urethra. It is located beneath the urinary bladder and above the rectum. It comprises five lobes, anterior and posterior lobes, two lateral lobes, and one median lobe. On the other hand, according to the embryonic origin, it can be divided into three glandular regions including, central zone (CZ), peripheral zone (PZ), and transitional zone (TZ) [60]. Moreover, there is an anterior fibromuscular stroma located at the anterior part of the prostate gland. These zones help clinicians to identify histological structures and pathological disorders. Microscopically, the prostate gland has two main cell types: luminal secretory and basal cells, which form a layer arrangement. The third cell type rare is the neuroendocrine cell [61]. The prostate produces prostatic fluid, a part of semen and provides prostatic acid phosphatase, proteolytic enzymes, zinc, fibrinolysin, and prostate-specific antigen for sperm viability [62].

Benign prostatic hyperplasia (BPH) and prostate cancer (PC) are common diseases found in the elderly. Particularly, PC is the second most worldwide deadliest cancer malignancy [63]. The incidence rate varies among ages and rises when patients get older. The incidence rate is nearly 60% when the ages over 65 years old. The mortality rate varies worldwide, but it rises with age, and almost 55% of all deaths occur >65 years old [64]. The aetiology of this disease remains unclear, but there was a report that said hormone (estrogen and androgen) and hormone receptors (androgen and estradiol receptor) involve the pathogenesis and progression of the PC [65]. In the past, animal models were a conventional way to study the pathophysiology of prostatic cancer. The mouse can apply in the study such as xenograft mice, nude mice, SCID mice, and transgenic mice (e.g. LADY and TRAMP) [66]. Researchers have an alternative way to study prostate cancer, including PC cell line (2D), PC spheroid (3D), and PC organoids (3D). To date, the most resembling method compared to in vivo study is prostate organoids.

Human prostate organoids can be derived from benign prostate tissue, PC tissue (primary, advanced or castration-resistant PC tissues), patients-derived xenograft

(PDXs) models, circulating tumour cells, embryonic stem cells, and iPSCs [58, 67, 68]. Drost and colleagues (2016) [69] demonstrated the successful cultivation, which composes of five main steps, including (1) dissecting tissue into small pieces (<1 mm^3), (2) tissue digestion by using enzyme (e.g. collagenase, trypsin), (3) resuspend the cell in the Matrigel, seeding the cell (40 ul/well), and wait 15 min to solidify Matrigel, (4) add the culture medium that specific for culturing prostate organoids as shown in Table 6.1, and (5) change the culture medium for 7–14 days or until the organoid is ready for being propagated. However, undefined and lot-lot variations of fetal bovine serum (FBS) may impact the experimental consistency and outcomes [70].

Current applications of prostate organoids for disease modelling and personalised medicine include identification of prostate cancer origins, gene mutation analysis and drug screening. The researchers demonstrated the genetic and signalling pathway manipulation on different prostate cancer origins resulting in different severity. The combination of c-Myc overexpression and PI3K/AKT activation cause high-grade prostate adenocarcinoma in basal cell-derived tumours, whereas it causes low-grade prostate adenocarcinoma in the luminal cell-derived tumours [74]. In Gao et al. [73]) study, they found that seven advanced PC organoid lines showed a similar pattern of genetic mutation, including *SPOP* mutation, *TMPRSS2-ERG* fusion, *SPINK1* overexpression and *CHD1* loss. Moreover, overexpression of histone methyltransferase enhancer of zeste2 (EZH2), an epigenetic modifier resulting in increased proliferation of prostate cancer [75]. These findings indicate how gene and signalling pathway activation is important for prostate cancer biology and may be useful for controlling the growth and progression of prostate cancer.

Although prostate organoids have several advantages, as mentioned, some limitations need to be improved. The stages of prostate cancer, including benign, advanced, or drug-resistance prostate cancer and the suitable culture conditions, directly affect the long-term culture of prostate organoids [69, 73].

Application of Reproductive Organoids in Personalised Medicine

Personalised medicine is a tailor-made or individualised treatment that promises to improve therapeutic approaches. Recently, researchers faced challenges in cancer treatment such as non-effective treatment and drug resistance. Therefore, using patient-derived organoids as a tool for drug screening has been progressively developed for providing specific and effective cancer treatment. Beltran et al. [76] demonstrated the promising clinical trial of alisertib, the aurora kinase A inhibitor, using a patient-derived prostate cancer organoid. The authors found that the cancer organoid responses to alisertib and showed the complex disturbance of aurora kinase A [76]. Besides, Girda et al. [77] demonstrated that the responsibility of endometrial organoids to the specific drug is dependent on the genetics of the patients. The author

Table 6.1 Summary of female and male reproductive organoids

Type of organoids	Cell source	ECM	Culture medium compositions	Key finding	References
Ovarian organoid	Ovarian cancer biopsies	Matrigel	Ovarian cancer organoid medium (OCOM) has 4 formula which adapted from endometrial organoids culture medium	Organoid derivation efficiency was low. Interestingly, the neuregulin-1 (NRG1) factor is a key to maximizing OC organoid growth and development	Maenhoudt et al. [14]
Fallopian tube organoid	Human fallopian tube	Matrigel	**Culture medium** – ADF medium – 25% conditioned mouse Wnt3A-medium – 25% conditioned mouse RSPO1 medium **Supplements** – 12 mM HEPES – 1% GlutaMAX – 2% B27 – 1% N2 – 10 ng/ml – human EGF – 100 ng/ml – human noggin, 100 ng/ml – human FGF-10 – 1 mM nicotinamide – 9 μM ROCK inhibitor (Y-27632) – 0.5 μM TGF-β RI Kinase Inhibitor IV (SB431542)	Fallopian tube organoids react to the *Chlamydial trachomatis* infection by increased production of LIF, which lowers the differentiated phenotype but increasing stemness potential. In addition, the bacteria also increases DNA hypermethylation, which indicates the molecular aging of the cell	Kessler et al. [25]

(continued)

Table 6.1 (continued)

Type of organoids	Cell source	ECM	Culture medium compositions	Key finding	References
	iPSC	**iPSC culture medium** – growth factor-reduced Matrigel **Differentiation** – Matrigel	**iPSC culture medium** – mTeSR®1 medium **Fallopian tube differentiation** Day 0–2 Primitive steak (PS) differentiation – Cell expose to 100 ng/ml human recombinant activin A and 3 μM CHIR99021 – DMEM/F12 – Glutamax – 500 U/ml penicillin streptomycin – 2% fetal bovine serum (FBS) – 10 μM ROCK inhibitor Y-27632 **Day 2–4 Intermediate mesoderm (IM) differentiation** – DMEM/F12 – Glutamax – 0.1 mM non-essential amino acids – 500 U/ml penicillin/streptomycin – 0.55 mM 2-mercaptoethanol – 10% Knockout Serum Replacement – 100 ng/ml BMP4 – 3 μM CHIR99021 – 10 μM ROCK inhibitor Y-27632 **Day 4–6 Müllerian duct (MD) induction** Fallopian tube medium (FTM) – DMEM/F12 – 500 U/ml penicillin/streptomycin – 2% reconstituted Ultroser G (15,950–017, Pall) – 10 μM ROCK inhibitor Y-27632 For Fallopian tube epithelium (TE) differentiation – 100 ng/ml human recombinant WNT4 with or without 3 μM CHIR99021 – 100 ng/ml human recombinant WNT3A with or without 3 μM CHIR99021 **Day 6–8** **FTM with supplement** – 20 ng/ml human recombinant Follistatin – 1 ng/ml estrogen – 33 ng/ml progesterone	This report establishes the fallopian tube epithelium organoids by using various chemical compounds. This model can be used as a disease-model of high-grade serous ovarian cancer (HGSC)	Yucer et al. [23]

(continued)

Table 6.1 (continued)

Type of organoids	Cell source	ECM	Culture medium compositions	Key finding	References
Endometrial organoid	Endometrial biopsies	Matrigel	– DMEM/F12 – Penicillin/streptomycin 1% – Glutamax 2 mM – B27 2% – N2 1% – Insulin-transferrin-selenium (ITS) 1% – Nicotinamide 1 mM – EGF 50 ng/ml – FGF10 50 ng/ml – Noggin 100 ng/ml – TGFβ/Alk inhibitorA83-01 0.5 μM – WNT activators WNT3A 200 ng/ml – RSPO1 200 ng/ml – N-acetyl-L-cysteine1.25 mM – p38 inhibitor SB202190 10 μM – 17β-estradiol (E2) 2 nM	This report was successful in establishing a long-term endometrial organoids cultivation. Moreover, the WNT/RSPO1 pathway is important for growth and expansion of endometrial organoids	Boretto et al. [31]
	Endometrial cancer tissue	Matrigel	**Expansion medium** – Advanced DMEM/F12 – N2 supplement 1X – B27 supplement minus vitamin A 1X – Primocin 100 μg/ml – N-Acetyl-L-cysteine 1.25 mM – L-glutamine 2 mM – Recombinant human EGF 50 ng/ml – Recombinant human Noggin 100 ng/ml – Recombinant human R-spondin-1 500 ng/ml – Recombinant human FGF-10 100 ng/ml – Recombinant human HGF 50 ng/ml – ALK-4, -5, -7 inhibitor, A83-01 500 nM – Nicotinamide 10 nM **Hormones used for differentiation of human endometrial organoids** – B-estradiol (E2) 10 nM – Progesterone (P4) 1 μM – 8-Bromoadenosine 3′5’-cyclic monophosphate (cAMP) 1 μM – Prolactin (PRL) 20 ng/mL – Human Placental Lactogen (hPL) 20 ng/mL – Human Chorionic Gonadotropin (HCG) 1 μg/mL	The significance characteristic of differentiated endometrial organoids in the mid-secretory phase is upregulation of genes *17βHSD2*, *SPP1*, and *LIF*	Turco et al. [8]

(continued)

Table 6.1 (continued)

Type of organoids	Cell source	ECM	Culture medium compositions	Key finding	References
	iPS cell line	**iPS culture medium** – Gelatin-coated dish **Differentiation** – ultra-low adherence plate	**iPS culture medium** – DMEM/F12 – 20% KnockOut Serum Replacement—1 mM nonessential amino acids – 2 mM GlutaMAX – 0.55 mM 2-mercaptoethanol – 10 ng/mL recombinant human FGF2 **Primitive steak (PS) differentiation (36 h)** – DMEM/F12 – 2 mM GlutaMAX – 100 IU/mL penicillin, 100 mg/mL streptomycin (P/S) – CHIR **Intermediate mesoderm (IM) differentiation (36 h.)** – DMEM/F12 – 2 mM GlutaMAX – 1 × P/S – 100 ng/ml FGF-2 – 1 mM retinoic acid **Coelomic epithelium (CE) induction (2 days)** – DMEM/F12 – 2.5% KnockOut Serum Replacement – 1 mM nonessential amino acids – 2 mM GlutaMAX – 0.55 mM 2-mercaptoethanol – 3 μM CHIR – 5 ng/ml NOGGIN – 10 ng/ml PDGF-BB **Müllerian duct (MD) induction (2 days)** – Basic differentiation medium – 3 μM CHIR – 5 ng/ml NOGGIN f **Endometrial stromal fibroblasts (EMSFs) culture medium (6 days)** – Basic differentiation medium – 1 μM 5aza2 – 3 μM CHIR – 10 ng/ml FGF-9 – 10 ng/ml PDGF-BB for 2 days – Followed by addition of 10^{-8} M E_2 for 4 days	The iPSC can be induced into defective endometrial stromal fibroblasts (EMSFs) cells which are related to endometriosis, uterine factor infertility, and endometrial cancer This can be done by differentiate iPSCs into PS, IM, CE, MD and EMSFs, respectively	Miyazaki et al. [32]

(continued)

Table 6.1 (continued)

Type of organoids	Cell source	ECM	Culture medium compositions	Key finding	References
Cervical organoid	Cervical cancer biopsy	Matrigel	– Advanced DMEM/F12 – 50 ng/ml human EGF – 250 ng/ml R-spondin 1 – 100 ng/ml Noggin – 10 mM Y27632 – 1 mM Jagged-1 – L-glutamine solution – Penicillin/streptomycin – Amphotericin B suspension	Cervical clear cell carcinoma (cCCC), an extremely rare disease of cervix showed a different genomic and histological analysis when compared to serous carcinoma (SC) which indicate that they have different pathogenesis	Maru et al. [41]
Trophoblast organoid	Early-term human placenta	Matrigel	– Advanced DMEM/F12 – N2 supplement – B27 supplement minus vitamin A – Primocin 100 μg/mL – N-Acetyl-L-cysteine 1.25 mM – L-glutamine 2 mM – Recombinant human EGF 50 ng/mL – CHIR99021 1.5 μM – Recombinant human R-spondin-1 80 ng/mL – Recombinant human FGF-2 100 ng/mL – Recombinant human HGF 50 ng/mL – A83-01 500 nM – Prostaglandin E2 2.5 μM – Y-27632 2 μM	Polarity of organoids is a main problem of gut-like organoids culture because orientations of the organoids affect the surface that attach to the Matrigel. This can be resolved by using suspension culture method which is successful in intestinal organoids	Sheridan et al. [51]
Testicular organoid	Testicular tissue	Extracted human testis ECM	**Testicular organoid formation media (ratio 8:1:1)** – Complete StemPro-34 – 30% FBS – 1 μg/mL solubilized extracted human testis ECM	The cultivation design for testicular organoid was successful with longfterm viability and morphological change at cell ratios 80% SSC, 10% Sertoli, and 10% Leydig	Pendergraft et al. [71]

(continued)

Table 6.1 (continued)

Type of organoids	Cell source	ECM	Culture medium compositions	Key finding	References
Prostate organoid	ES cell	**hESC colony culture** – Matrigel **Induction of definitive endoderm (DE)** – No **Prostatic fate determination** – No **Prostate organoid growth and differentiation** – Matrigel	**hESC colony culture** – mTeSR1 medium **Induction of definitive endoderm (DE) (3 days)** – RPMI 1640 media – 100 ng/ml activin A – 2 mM L-glutamine – 100 U/ml penicillin—100 μl/ml streptomycin – Daily increasing defined fetal bovine serum at concentrations of 0, 0.2 and 2% **Prostatic fate determination (4 days)** – RPMI 1640 containing 2 mM L-glutamine – 500 ng/ml human FGF10 – 500 ng/ml human WNT10B in and 100 U/ml penicillin—100 μl/ml streptomycin **Prostate organoid growth and differentiation** – Collect organoid into Matrigel Basement Membrane Matrix Growth Factor Reduced, Phenol Red-Free containing 1X B27 supplement, 100 ng/ml Noggin and 100 ng/ml EGF – **Add into plate and solidification** – **Culture in media containing** 1:2 prostate epithelial cell growth medium (PrEGM) and stromal cell basal medium (SCBM) supplemented with 2 mM L-glutamine, penicillin–streptomycin, 15 mM HEPES, 500 ng/ml R-Spondin1, 100 ng/ml Noggin, 100 ng/ml EGF, 1X B27 supplement, 10 nM ATRA, and 1.7 μM testosterone	– Exposure time of WNT10B/FGF10 is important for protatic formation – Bisphenol-A (BPA), an endocrine disruptor can be used as a prostate carcinogenesis model with aging	Calderon-Gierszal and Prins [68]

(continued)

Table 6.1 (continued)

Type of organoids	Cell source	ECM	Culture medium compositions	Key finding	References
	iPS cell	**Human iPSC generation** – Matrigel **Definitive endoderm induction** – Matrigel **Coculture of human iPSCs with rat urogenital sinus mesenchyme (UGMS) cells** – GFR-Matrigel	**Human iPSC generation** – mTeSR1 medium **Definitive endoderm induction** – DMEM/F12 medium – 100 ng/mL Activin A – Daily increasing defined fetal bovine serum at concentrations of 0, 0.2 and 2% **Coculture of human iPSCs with rat UGMS cells** – ADMEM/F12 containing – EGF 5–50 ng/ml – 500 ng/ml recombinant R-spondin1 – 100 ng/ml recombinant Noggin – TGF-/Alk inhibitor A83-01 – 10 ng/ml FGF10 – 5 ng/ml FGF2 – 1 μM Prostaglandin E2 – SB202190 (10 μM) – 10 mM Nicotinamide – Dihydrotestosterone at 0.1–1 nM final concentration	In human prostate cultivation, it is frequently outgrowth of basal epithelial cells. UGMS tissue recombination assays can generate prostate tissue with luminal cells which can give rise to basal cells	Hepburn et al. [58]
	PDXs	Low attachment plate	Basis medium containing – 10 μM Y-27632-HCl – 5% fetal calf serum – 1 × B-27 supplement – 10 mM Nicotinamide – 500 ng/ml R-spondin – 1.25 mM N-acetyl-cysteine – 10 μM SB202190 – 100 ng/ml Noggin – 500 nM A83-01 – 10 nM Dihydrotestosterone DHT – 10 ng/ml Wnt3A – 50 ng/ml HGF – 50 ng/ml EGF – 10 ng/ml FGF10 – 1 ng/ml FGF2 – 1 μM PGE2	PDX-derived organoids show genomic similarity to primary tumors which are beneficial for drug screening tests	Karkampouna et al. [72]

(continued)

Table 6.1 (continued)

Type of organoids	Cell source	ECM	Culture medium compositions	Key finding	References
	Prostate metastasis cell and circulating tumor cell	- GFR-Matrigel	– Advanced DMEM/F12 – EGF 50 ng/ml – R-spondin 15% v/v – Noggin 10% v/v – FGF2 1 ng/ml – FGF10 10 ng/ml – Dihydrotestosterone (DHT) 0.1 nM for CRPC samples and 1 nM for hormone-sensitive samples – Nicotimanide 10 mM – A83-01 0.5 μM – Y-27632 10 μM – B27 1x – N-acetyl-L-cysteine 1.25 mM – Glutamax 2 mM – HEPES 10 mM Primocin 1:100 v/v	Prostate organoids provide a great opportunity to define genetic alteration that link to progression and metastasis of the disease	Gao et al. [73]

discovered in their study that Napabucasin, the STAT3 transcription factor inhibitor, strongly inhibits the growth of all cultures of patient-derived endometrial organoids. Meanwhile, fluvestrant, a selective estrogen receptor degrader affects only some cultures of patient-derived endometrial organoids.

These examples demonstrated that the patient-derived reproductive organoids are feasible for developing drug screening tests for individual cancer patients. Moreover, the organoid model is necessary for clinical trial evaluation and decision making.

Conclusion

Reproductive organoids can be generated from adult stem cells, which is established directly from the healthy or pathogenic tissue of reproductive organs or pluripotent stem cells, which latter is being differentiated into the reproductive cells. The information regarding human reproductive organoids discussed in this chapter emphasised the advantages of applying the organoids in modelling human infectious diseases, genetic disorders, and cancers that cause reproductive organ problems, as summarised in Table 6.1. Moreover, patient-derived reproductive cancer organoids are currently developed for biobanking. The organoid biobanking will further assist drug development platforms and personalised medicine.

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Chapter 7
Production and Application of Mesenchymal Stem Cell Spheroids for Cartilage and Bone Regeneration

Ngoc Bich Vu and Phuc Van Pham

Abstract **Introduction**: Cartilage and bone tissues are widely utilized in regenerative medicine, especially in treating injured cartilage and bone. Some attempts were made to produce these tissues from stem cells and scaffolds for a long time, combined with some growth factors and inducible factors. However, owing to the complexity of cartilage and bone tissues, these efforts generated limited results. This chapter explores the production and usage of mesenchymal stem cell (MSC) spheroids in cartilage and bone regeneration. **Methods**: The data regarding production and applications of mesenchymal stem cell spheroids for cartilage and bone regeneration were searched in the PubMed, Web of Science, and Google scholar databases with the keywords "derived mesenchymal stem cell spheroids", "cartilage regeneration", and "bone regeneration". **Results**: The formation of cartilage or bone tissue through the use of MSC spheroids mimics the formation of cartilage and bone during embryogenesis. These microtissues can be directly used as materials for transplantation or building blocks to generate the cartilage and bone macrotissues. **Conclusion**: Based on our studies and recent publications, we support the production and application of microtissues of cartilage and bone from mesenchymal stem cell spheroids for cartilage and bone regeneration.

Keywords Bone engineering · Cartilage engineering · Microtissues · Mesenchymal stem cells · Tissue engineering · Regenerative medicine

Abbreviations

ADSC	Adipose-derived stem cell
BM	Bone marrow

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ECM Extracellular matrix
MSC Mesenchymal stem cell

Introduction

Bone and cartilage defects are common clinical diseases that can significantly affect patients' quality of life. Numerous efforts have been made to treat these defects in the long term. Owing to the progress of stem cell research, some new therapies based on stem cells have been developed for 20 decades. Some reports showed that the transplantation of stem cells (as cell suspension) generated promising results for cartilage [1–5] and bone regeneration [6, 7]. Some stem cell-based products were developed and commercialized to treat cartilage injury [8, 9]. However, the current therapies still contain some issues that need resolution. Indeed, the treatment efficacy is limited in the case of large and deep damages. In order to overcome these limitations, there have been some novel efforts, such as the transplantation of stem cells in the form of spheroids [10–12], in the form of sheets [13, 14], or combination with biomaterials [15, 16].

The stem cells in spheroids also referred to as 3D cultures of stem cells, are cultured in nonadherent conditions to connect and form cellular aggregates. In 3D conditions, stem cells mimic their natural state in the human body to display characteristics different from those seen in the 2D culture. This chapter aims to introduce the state-of-the-art technologies used to culture MSC spheroids and highlight some of their applications in bone and cartilage regeneration.

Mesenchymal Stem Cell Spheroids

Mesenchymal Stem Cells

Mesenchymal stem cells are adult stem cells found in almost all vascular tissues in human beings. They were discovered more than 40 years ago by Friedenstein [17]. For the first time, Friedenstein cultured the bone-forming cells from a guinea pig [17]. In 1988, Owen et al. re-created this experiment on rats [18]. Following that, in 1992, MSCs from human bone marrow were first isolated and cultured [19]. The first clinical application of MSCs was carried out in 1993 (and reported in 1995). In this clinical study, Lazarus et al. obtained bone marrow samples from 23 patients with hematologic malignancies. MSCs were successfully cultured in 15 out of the 23 bone marrow samples for 4–7 weeks. These MSCs were autologously infused to patients. No adverse reactions were observed in any of the transplantations [20].

MSCs can be detected and isolated in various human body tissues, such as bone marrow, adipose tissue, peripheral blood, menstrual blood, umbilical cord, umbilical

cord blood, placenta, and milk. Although the MSCs from different tissues display some differences in their biological characteristics, they also are defined as MSCs as per the criteria suggested by Dominici et al. and the International Society of Cell and Gene Therapy (2006). These criteria included that they (1) adhere to the culture vessel surface in the standard culture condition, (2) express the CD73, CD90, and CD105 and lack expression of CD11b or CD14, CD34, CD45, CD79-alpha or CD19, and HLA-DR, (3) be induced into adipocytes, osteoblasts, and chondroblast in vitro [21].

The therapeutic potential of MSCs relates to their capacity to perform differentiation to produce mesoderm-derived cells and bring about transdifferentiation in some kinds of ectoderm- or endoderm-derived cells. MSCs own strong immune modulation and angiogenesis. It appears that utilizing immune modulation, MSCs were rapidly enrolled in the biotechnological industry with some MSCs-based stem cell drugs. The first stem cell drug (Prochymal) was approved in Canada in 2012 based on the aforestated characteristic as a mechanism to treat graft versus host disease (GVHD) [22]. Some recent studies have shown that MSCs may join in angiogenesis through different ways, significantly secreted factors [23–26]. Due to this potency, MSCs transplantation was used to treat more than 100 diseases and health conditions, some extremely serious (according to https://www.clinicaltrials.gov and review reports) [27, 28]. Even more, biological characteristics have been discovered, which guide the tissue engineering and regenerative medicine applications. MSC transplantation is becoming one of the most popular kinds of stem cell transplantation. Moreover, MSCs-based drugs have been developed, which stimulated and boosted the clinical usage of MSCs [29].

Besides applying MSCs in stem cell therapy, MSCs also constitute the basic materials for tissue engineering, especially in engineered cartilage and bone tissues. MSCs are one of three essential materials of tissue engineering. They can produce some engineered tissues such as cartilage and bone combined with other materials and signalling factors. However, MSCs used in this engineering are complex with some different strategies that MSCs can load in the scaffold as single cells, spheroids, or pellets.

Mesenchymal Stem Cell Spheroids

MSC spheroids are cellular structures in the form of cell aggregates that have physiological links between cells inside the frames. Owing to their differences from cell pellets or cell clumps that link cells together by mechanical forces or spontaneously, MSCs in spheroids are linked together by extracellular matrix proteins after a certain period to form three zones within the spheroid. Therefore, MSC spheroids or cell spheroids only can be formed by organogenesis cultures. It seems that the definition of spheroids was used from a procedure explained in oncology where cancer cells can form certain structures named tumorspheres or spheroids. Structurally, MSC spheroids contain two or three zones depending on the culture conditions (outer zone,

middle zone, and necrotic core). Experimentally, MSC spheroids usually include two zones: outer zone and inner zone. MSCs in the inner zone deal with more hypoxic and have fewer nutrients than those in the outer zone. Moreover, the MSCs in the inner zone is surrounded by other MSCs, whereas those in the outer zone are partly covered with ECM and other cells (Fig. 7.1).

Some studies have shown that there are many differences in the biology of MSCs cultured as a monolayer (2D) and the ones cultured as spheroids (3D). First, the size of MSCs is reduced compared to the monolayer culture; some reports showed that the size of MSC reduced up to 75% [30–32]. The cell properties of MSCs inside spheroids are also affected by the mechano-physical differences between the 2D and 3D cultures. In the 2D platform, MSCs will adhere to the surface of culture vessels

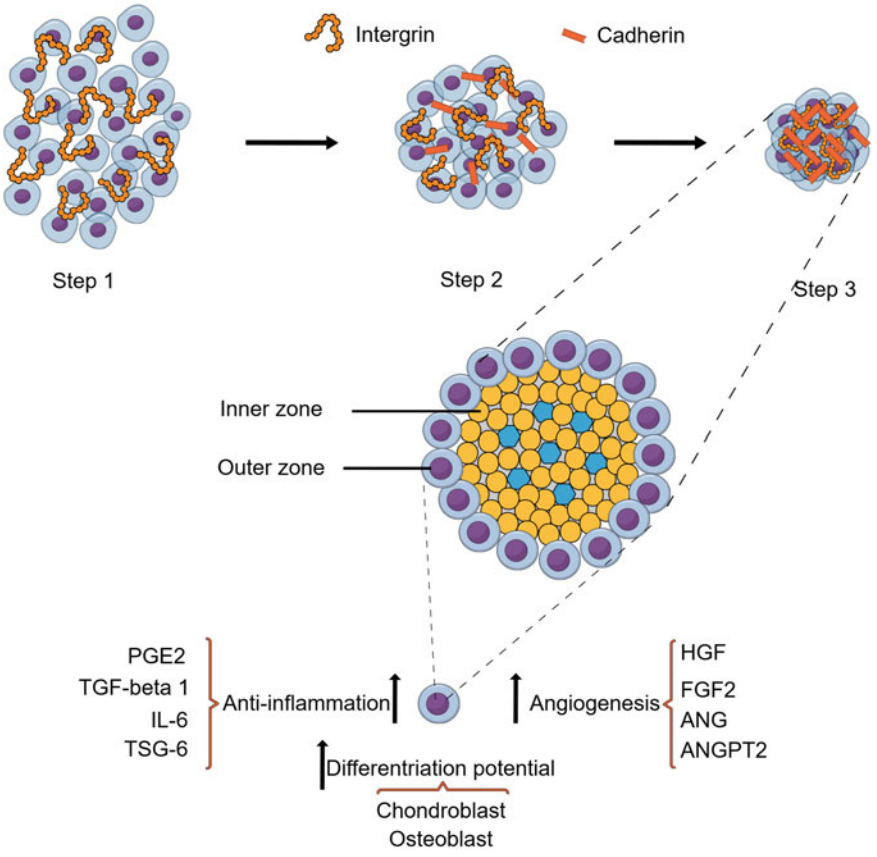

Fig. 7.1 The formation and structure of MSC spheroids. Through the 3-step process MSC spheroids were formed with inner zone and outer zone. In the form of spheroids, MSCs up-regulated the production of some anti-inflammation cytokines (PGE2, TGF-beta 1, IL-6 and TSG-6), angigenic factors (HGF, FGF2, ANG, and ANGPT2), and differentiation potential toward chondroblasts and osetoblasts

and expose the remaining parts to the culture medium. However, in the 3D platform, MSCs are stuck in ECM and the cells around it. Indeed, Young's elasticity modulus of the materials surrounding the cells causes some differences in cell physiology properties, especially cell differentiation [33, 34]. The plasticity modulus was recorded as a massive point of differentiation between 2 and 3D cultures; in the 2D culture, the plasticity modulus reaches some gigapascals (Gpa), which is less than 0.1 kPa in 3D culture [32]. These differences alter the epigenetics of MSCs in spheroids.

Potapova et al. showed that MSCs in 3D cultures upregulated 1731 genes and down-regulated 1387 genes compared to 2D cultures [35]. Almost all the upregulated genes in MSCs in 3D cultures were related to hypoxia, angiogenesis, and inflammation [35]. In the 3D platform, MSCs also saw an increase in their immune modulation potential. Bartosh et al. observed that MSCs upregulated the TNAIP6/TSG6 in the form of spheroids [31]. The angiogenic potential is also enhanced in MSC spheroids. The angiogenesis-related genes such as angiogenin, FGF2, HGF, and VEGF are upregulated in MSCs in the form of MSC spheroids [35–37].

Interestingly, the chondrogenic differentiation potential of MSCs is enhanced in the MSC spheroids. In their observation, Johnstone et al. discovered that under the same inducible conditions, MSC spheroids could be more efficaciously induced to chondrocytes by bringing about an increase in the alkaline phosphatase activity, both in type IIA and IIB collagen (at mRNA level) [38]. Similarly, Yoo et al. also noted the same in bone marrow-derived MSC spheroids with regard to the expression of type I, type II, and type X collagen in MSC spheroids. Significantly, these authors also found that aggrecan and link proteins are expressed in the extracts of cell spheroids [39]. Numerous recent studies have confirmed this [40–42].

Moreover, MSC spheroids also significantly increased markers of osteogenic differentiation [34, 43–45]. This was observed by Yamaguchi et al. in rat MSC spheroids for osteogenesis in vitro and in vivo. In vitro, in the same condition of osteogenesis, the calcium deposition is better in MSC spheroids than in the monolayer. In vivo, the results showed that MSC spheroids could participate in bone regeneration better than the MSC monolayer in rat calvarial defect models [43]. In a recent study, Kim and Adachi showed that in the inducible medium, MSC spheroids could form the osteocyte likeness only within two days after being induced compared to the conventional 2D culture. The study suggested that the cell-condensed condition of spheroids decided the differentiation fate of MSCs toward osteocytes [45].

Methods for Mesenchymal Stem Cell Spheroid Production

Some methods can be used to form MSC spheroids from MSCs. It appears that there is an absence of a special medium to produce MSC spheroids. Indeed, in almost all the methods, MSC spheroids can be produced in the 2D culture in the case of serum-based media. However, the effects of culture media on MSC spheroid formation were also investigated. Owing to the fact that in almost all studies, MSC spheroids were produced in fetal bovine serum-based media, there were certain safety issues

such as prion exposure risk, toxicological risk, and immunological risk when the obtained spheroids were used in clinical applications [46, 47]. Therefore, xeno-free media, as well as chemically defined media, are developed to expand MSCs. Ylostalo et al. showed that some commercial media could not support the compact spheroid formation, while the medium supplemented with human albumin serum could facilitate the formation [48].

In contrast, Domnina et al. produced MSC spheroids from endometrial MSCs using the serum medium [49]. In another effort to use serum-free media to produce MSC spheroids, Zhao et al. attempted to use the TeSR-E8 medium (a chemically defined serum-free medium for pluripotent stem cells) to make MSC spheroids. The results showed that MSC spheroids could form in the medium following three to five days of culture [50].

MSCs from some different sources were used to produce MSC spheroids. MSCs from bone marrow [51, 52], adipose tissue [53, 54], gingiva tissue [55], endometrial tissue [49], umbilical cord tissue [56], and dental pulp tissue [57] can form spheroids in suitable conditions. The differences in spheroid formation capacity between the different kinds of MSCs are not yet thoroughly studied. Fennama et al. compared the MSC spheroid formation from bone marrow tissue with the MSC spheroid formation from adipose tissues and SVF. It was found that BM-MSCs can form the compact spheroids as can ADSCs, to are a lesser extent, while SVF showed poor spheroid formation [58].

The spheroids from MSCs can be formed through a three-step process. This included (1) the loose contact and interaction between cell-cells by integrin and ECM to form the loose cellular aggregates; (2) enhancement of cadherin expression by cells inside aggregates to make them into more compact and condensed aggregates; (3) formation of compact spheroids by the tight junctions of cadherin-cadherin between MSCs [59] (Fig. 7.1). In addition, Robinson et al. (2003) demonstrated that the expression of cadherin and integrin play significant roles during spheroid formation [60].

As stated, the first step in the spheroid formation process is highly crucial for the initiation of spheroids. All the present methods appear to introduce ways to help MSCs contact and interact using their integrins in cell surfaces before triggering the overexpression of cadherin in the next step. Some developed techniques to form spheroids include the hanging drop, nonadherent surface, spinning flask, rotating culture vessels, external force-assisted, and matrix-embedded methods (Fig. 7.2). These methods have their own advantages and disadvantages. Hanging drop is the most popular method to produce standard spheroids by the natural cells-cells link inside spheroids. However, this method is so costly, and it is so difficult to scale up.

Hanging Drop

The hanging drop technique has been well-established for a long time. This technique was developed and used in microbiology to produce media that can be maintained for drops with minimal evaporation without spreading. Subsequently, this technique

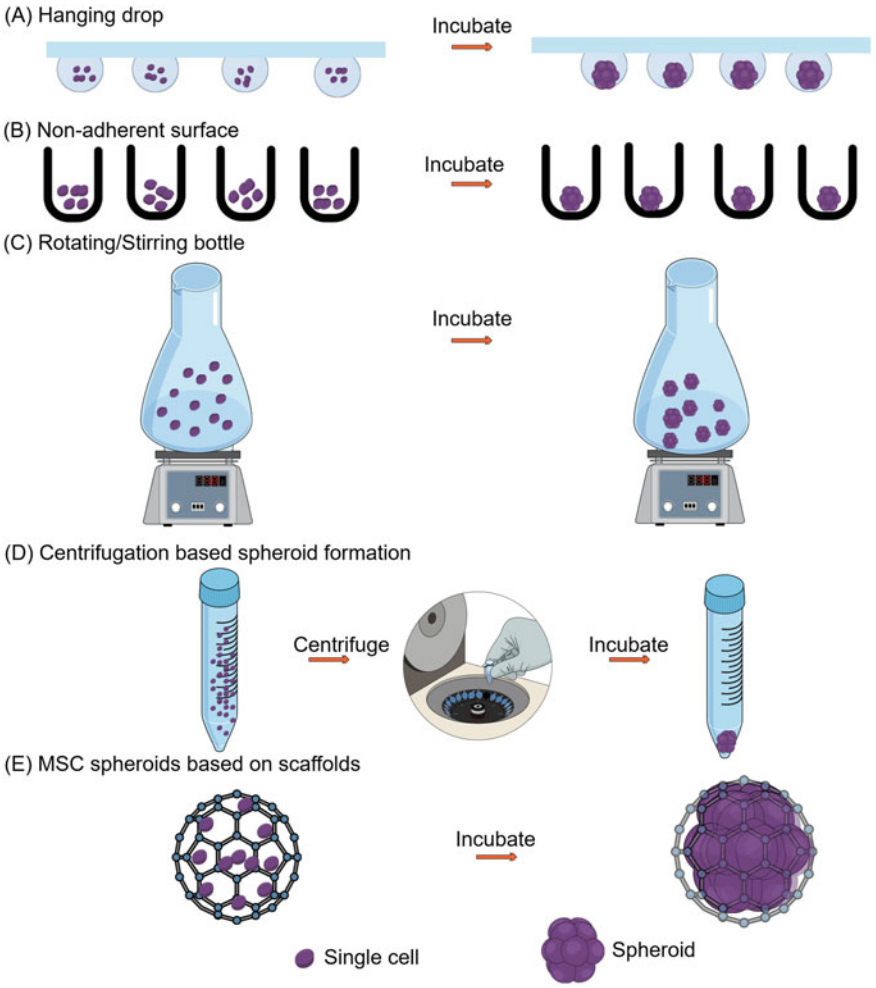

Fig. 7.2 Some current methods used to produce MSC spheroids. **a** hanging drop, **b** using non-adherent surfaces, **c** using rotating or stirring bottle, **d** centrifugation based spheroid formation, and **e** MSC spheroids based on scaffolds

was used in animal and human cells in neural tissue culture during the twentieth century [61, 62]. Moreover, this technique is now employed to culture stem cells [63–65] and form embryoid bodies from embryonic stem cells or pluripotent stem cells. The principle of this technique is simple: a cell suspension with 300–3000 cells per 15–40 uL of medium is deposited onto the underside of the lid of the tissue culture dish/plate. Then, the lids are inverted, and drops are held on the lid by surface tension. Affected by gravity, free cells in the drops can concentrate and facilitate the making of the loose aggregates. This technique permits controlling the size of spheroids as well as cell numbers. This technique can be easily applied by

special plates such as Perfecta3D 96-well hanging drop plates and crystallization plates. These consumables help make the hanging drop technique more accessible and reproducible.

Nonadherent Surface

This technique appears to be the simplest as it does not require additional equipment. MSC spheroids can be easily produced by using the plate or petri dish. Some commercial non- or low-adhesive plates or dishes are developed and commercialized, or simply, the culture plates can be coated with an agarose thin film, hydrophobic polymers, or lactonamide. In general, the MSC spheroids obtained from this technique are uniform in spheroid size and shape. In some cases, spheroids do not display a sphere-like shape.

Recently, to improve the shape and structure of MSC spheroids, some novel technologies have been applied to produce nonadherent microwells. Using this technology, the size and shape of spheroids can be controlled by the size of microwells. The microwells can be fabricated using microscale techniques by micro-moulding cell-nonadhesive inert materials such as agarose or polyethylene glycol (PEG). The MSC suspension would be loaded into the fabricated device, and the cells would be automatically assembled to make the spheroids.

Rotating Bioreactors

Spinner flasks or roller bottles can be used in this technique. The diameter of the spheroids can be controlled based on the cell density, medium composition, spinning rate, and culture time. Unlike the hanging drop method or the nonadherent surface where the cells are cultured in the static condition, MSCs exist in a dynamic condition. Therefore, in this platform, MSCs will face a strong shear force that can affect their physiology.

Formation of Spheroids Using External Force-Assisted Methods

This strategy uses some external forces to make cellular aggregates from the cell suspension. The simple way is low-speed centrifugation. Other methods such as dielectrophoresis, magnetic fields, and ultrasound standing wave traps also are utilized to produce spheroids. These strategies aim to enhance the first step of the spheroid formation process. After making the cell aggregates through external forces, the cell aggregates are cultured in suitable conditions to boost the second and third steps that make the compact spheroids.

Producing MSC Spheroids Based on Scaffolds

This technique uses a specific type of material and MSCs to produce MSC spheroids. Hydrogels and inert matrices are popularly used in this technique. The hydrogels contain a network of cross-linked polymer chains that can absorb and retain water. In addition, the inert matrices include sponge-like membranes made of polystyrene to create pores that permit MSCs to bind and increase inside. Using hydrogels (Matrigel, alginate, and Qgel Matrix), MSCs should be mixed with hydrogels, and then these complexes should be seeded on suitable surfaces to leave the spheroid formation.

Productions of Bone and Cartilage Microtissues from Mesenchymal Stem Cell Spheroids

Production of Cartilage Tissues from MSC Spheroids

The first report on employing MSC spheroids to make engineered cartilage was written by Anderer and Libera in 2002. To date, many publications have used this strategy to produce cartilage tissue. It seems that all approaches to make MSC spheroids can be utilized to make cartilage microtissue. In the first report, Anderer and Libera used the nonadherent surface to enhance the aggregate formation. In this study, wells of plates were coated with agarose 2% to inhibit the attachment of cells to the well surface to facilitate the aggregates via the integrin interaction [66]. Markway et al. successfully produced cartilage tissues from MSC spheroids. In this study, the centrifuge was used to condense 2×10^5 cells into cell pellets. Then, the cell pellets were induced into the cartilage using the chondrogenesis medium [67]. In a recent publication, Vu and Nguyen et al. developed an easy and scalable method of producing MSC spheroids for cartilage and bone tissue engineering [68]. The method used the V-bottom 96-well plates to facilitate the formation of cell aggregates. In another report, Tu et al. used the hanging drop method to produce MSC spheroids and then induced them to cartilage. The MSC spheroids were induced for 21 days, and they strongly expressed the cartilage phenotype, including aggrecan, glycosaminoglycan, and type II collagen. They also expressed some chondrogenic genes at the mRNA level, including Sox9, Col2, Col1, and Acan [69].

In order to create cartilaginous macrotissues, Le et al. loaded MSC spheroids produced from the hanging drop method onto the porous scaffold. The complexes of MSC spheroids and scaffold expressed the cartilage phenotype after induced chondrogenesis for 21 days [56]. In addition, these structures were positive with Safranin O staining, alcian blue staining, and collagen 2, with significant expression of Sox9, Col2, and aggrecan at mRNA levels [56]. More interestingly, these complexes could become mature cartilage after their transplantation into animals [56]. In another report, De Moor et al. successfully produced engineered cartilage from MSC spheroids. In the first step, the MSC spheroids were created using

microwells; then, these spheroids were induced in the chondrogenesis medium for 42 days at 5% oxygen. Chondrogenic spheroids were subsequently used to produce the macrotissue using bioprinting [70].

Production of Bone Tissues from MSC Spheroids

In an early study, Cerwinka et al. produced MSC spheroids from BM-MSCs with a porous gelatin scaffold [71]. Then, these MSC spheroids were induced toward osteogenesis using the osteogenic medium for 10 days. After 10 days, the spheroids exhibited the bone phenotype with strong calcification, alkaline phosphatase expression, and the presence of vitamin D receptor [71]. Similarly, Laschke et al. produced MSC spheroids from ADSCs in polyurethane scaffolds; then, they were successfully induced into bone microtissues that were strongly positive with Kossa staining [72].

In 2017, Lee et al. investigated the osteogenic potential of spheroids that were made from gingiva-derived MSCs and osteo-precursor cells using the concave microwell technique [55]. These spheroids expressed the bone tissue phenotype after Day 5 of culture. Indeed, they were positive with alkaline phosphatase, deposited mineralized extracellular matrix, and positive with Alizarin red staining [55]. Fennema et al. compared the osteogenic potential of MSC spheroids made from ADSCs and those made from BM-MSCs in mice. The authors showed that both (spheroids from BM-MSCs and ADSCs) could form ectopic bones in mice [58].

In 2018, Tae et al. investigated the osteogenic potential of MSC spheroids made from a mixture of two kinds of MSCs (gingiva and bone marrow-derived MSCs). The authors mixed gingival MSCs and BM-MSCs at specific ratios and produced spheroids using the concave microwell technique. The in vitro analysis showed that the spheroids enhanced the expression of alkaline phosphatase and increased the expression of Runx2 and osteocalcin in mRNA [73]. Recently, Aguilar et al. successfully produced the microtissue of bone using MSC spheroids [74]. In their study, MSC spheroids were produced to make the aggregates by using a low-speed centrifuge. Then, they were differentiated into bone microtissue by incubation in the osteogenic medium for 28 days. Following induction, the cells inside the spheroids exhibited a robust osteogenic response to the differentiation medium, including a higher mRNA of alkaline phosphatase, collagen type I, and osteocalcin compared to the levels noted before differentiation. The authors suggested that the centrifugation method is more effective than gravity for producing MSC spheroids [74].

In an effort to make larger constructs, spheroids were used as building blocks. Ahmad et al. successfully made large bone constructs using MSC spheroids. In the first step, MSC spheroids were made from ADSCs and poly(L-lactic acid) nanofibers. The interaction of MSCs with nanofibers that triggered ADSC osteogenesis could be detected by expressing osteocyte markers after being cultured in a medium without osteogenic factors for seven days. The large bone constructs were created using these spheroids as building blocks [75]. Heo et al. produced the bone tissue by utilizing this approach. They produced spheroids from both MSCs and human umbilical vein endothelial cells. These spheroids were then induced for 10 days to create microtissues of bone before they were used to produce large bone constructs using aspiration-assisted bioprinting in combination with hydrogel. These bio-printed bone tissues exhibited interconnectivity with the actin-filament formation and a high expression of genes related to osteocytes and endothelial cells [76].

Applications of MSC Spheroids and MSC Spheroid-Derived Cartilage in Bone Regeneration

To date, published studies have shown that both MSC spheroids and cartilages from MSC spheroids are used in animal models to treat cartilage and bone injury. Sekiya et al. produced osteochondral defects in rabbit models and treated defects through the use of synovium-derived MSC spheroids [77]. After 12 weeks, MSC spheroids were found to have attached to the osteochondral defects and were strongly positive with Le et al. produced MSC spheroids from ADSCs using the hanging drop technique. These MSC spheroids were loaded into the porous scaffolds by employing low-speed centrifugation. The complexes of MSC spheroids-scaffolds were induced using chondrogenesis by an inducible medium before they were used to treat cartilage damage on the hindlimb knee in rats [56]. After 12 weeks, the results showed that the cartilage that was formed at the defects was strongly positive with Safranin O staining [56]. Zhang et al. used the functionalized scaffold for in situ TGF-beta 1 transfection to produce MSC spheroids, these spheroids became mature cartilage after 8 weeks of transplantation in animals [78].

In the rat calvarial defect model, Yamaguchi et al. showed that the transplantation of MSC spheroids could enhance bone regeneration, recorded by micro-computed tomography and histological analysis [43]. Suenaga et al. treated the calvarial defects in rats using MSC spheroids [10]. The authors compared the treatment efficacy of transplantation of MSC spheroids, beta-tricalcium phosphate, and a combination of beta-tricalcium phosphate with MSC spheroids. The results confirmed that only MSC spheroids could support bone regeneration in the defects [10]. Murata et al. treated osteochondral defects in pigs using MSC spheroids from ADSCs in the big animal models. In this trial, MSC spheroids were created on a nonadherent surface using

the 96-well plates. Each MSC spheroid contained 5×10^4 cells. Following this, 760 spheroids were placed in a cylindrical mould to develop macrostructures that would fit the pigs' defects. The macrostructures were cultured for seven days to create links between spheroids before they were transplanted into the pigs. After 12 months, the osteochondral defects were regenerated into the original structure of cartilage and subchondral bone [79]. Murata's group repeated this study with more pigs [12]. The results confirmed that the transplantation of scaffold-free MSC spheroids from ADSCs successfully induced regeneration of hyaline cartilage and subchondral bone structures over 12 months [12].

Yanagihara et al. used genetically modified MSCs to create MSC spheroids [11]. In this study, MSCs enhanced the expression of Runx2 to stimulate osteogenesis; then, they were transplanted into bone defects on fat femurs. The results showed that MSC spheroids boost bone regeneration more significantly than MSCs from the monolayer culture [11]. Similarly, Moritani et al. found the same results when the effects of periodontal ligament-derived MSCs and MSC spheroids in the treatment of murine calvarial defect models [80] were compared. MSCs in the form of MSC spheroids also function better in bone regeneration than MSCs in the form of cell suspension.

In a recent study, Findeisen et al. used MSC spheroids to treat critical-sized bone defects in nude mice. The micro-CT analysis showed that the bone material density is significantly higher in the group with MSC spheroid transplantation than in MSCs [81].

Cartilage organoid for personalized treatment.

Conclusion

Bone and especially cartilage damages are popular injuries with low self-regeneration. For a few decades, stem cell transplantation has been used as a novel approach to stimulate cartilage and bone regeneration with promising results. However, a recent publication showed that the application of stem cell transplantation showcased low efficacy in case of large and severe damages.

This chapter summarizes some methods to produce spheroids as well as the usage of these spheroids in bone and cartilage regeneration. Although there has been no clinical usage of MSC spheroids in cartilage and bone regeneration, the in vitro and preclinical data from animals suggests that MSC spheroids, with or without differentiation, are promising building blocks that can be used, directly or via the production of macrotissues, for bone and cartilage regeneration.

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Chapter 8
Biomaterials in Organoid Development

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Abstract **Introduction**: The animal model and the traditional two-dimensional (2D) cell culture have long been used to understand the biology and pathology of cell behaviour. However, neither technique captures precise cell behaviours, such as the in vivo cell-cell and cell-extracellular matrix (ECM) interactions and intra- and interorgan interactions. Organoids are three-dimensional cell culture systems known to simulate many of the structural and functional features of the organ. The microenvironment and signals within the body profoundly affect the development of stem cells in vitro and in vivo. Organoid culture matrices range from naturally derived to synthetic biomaterials with varying biophysical properties. This chapter focuses on the regulation of cell-matrix interactions that direct the decision of stem cells, including the various types of biomaterials used for the reproducible generation and control of organoid cultures. **Methods**: A few databases, such as Google Scholar, PubMed, and Scopus, were used to select literature with keywords organoids; extracellular matrix; cell interaction and regulation; biomaterials; natural and synthetic matrices. **Results**: Organoids provide a reliable tool for a wide range of disease modelling and a potential drug screening and toxicity testing strategy. However, it is difficult to control stem cell fate to promote proliferation and differentiation into specific cell types. The stem cell fate is determined by many factors, particularly the appropriate matrices required for multiple stages of organoid development and subsequent in vivo propagation. **Conclusion**: Organoids can be used to investigate human physiology in vitro, but their maturation depends on the stem cells' capacity to form highly organised structures. Stem cell development and essential design parameters for organoid matrices are affected by various material features,

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including the presentation of cell-binding ligands, matrix dynamics, structural geometry, and degradability. Three-dimensional (3D) ECMs can be tailored to optimise the numerous structural and metabolic characteristics that influence cell fate.

Keywords Organoids · Biomaterials · Extracellular matrix · Matrices

Abbreviations

2D	Two-dimensional
ECM	Extracellular matrix
3D	Three-dimensional
EGF	Epidermal growth factor
EHS	Engelbreth-Holm-Swarm
PEG	Poly(ethylene glycol)
PCL	Polycaprolactone
FDA	Food and Drugs Administration
EMA	European Medicine Agency
GMP	Good manufacturing practices
PLGA	Poly(lactide-co-glycolide) or poly(lactic-co-glycolic acid)
LA	Lactic acid
GA	Glycolic acid
HA	Hyaluronic acid
hPSC	Human pluripotent stem cell
ADSC	Adipose-derived stem cells
HLO	Human lung organoids

Introduction

Human disease and treatment have relied mainly on in vitro cultures and animal models. Traditional in vitro models, cultured on two-dimensional (2D) plastic surfaces, have advanced the understanding of biology and pathology. However, the cell behaviour differs significantly from their in vivo counterparts, and the models do not capture in vivo cell-cell and cell-extracellular matrix (ECM) interactions and intra- and interorgan interactions. On the other hand, animal models allow for testing in a living system, but they are costly and time-consuming. Human organs are complex networks with physical (matrix microstructures and stiffness), mechanical (fluidic forces and mechanical strain), and biochemical (growth factors and cytokines) properties. These structural and physiological features have a significant impact on organ development and function. Organoids are spherical aggregates of cells that form as a result of spontaneous differentiation of adult or pluripotent stem

cells in vitro in a three-dimensional (3D) space. Organoids imitate the corresponding organ in normal, developmental or disease model systems, providing a foundation for in vitro modelling of organ and tissue properties [1, 2].

In contrast to 2D cell culture systems, the organoid cultures offer the possibility to learn the features of organs and tissues in 3D models that closely resemble human physiology and capture tissue diversity while providing a high level of accessibility and tractability, which are impossible to achieve in vivo [3]. Multiple methods for organoid developments have been described. However, the typical pattern includes proliferation, differentiation, cell sorting, lineage commitment and morphogenesis [4, 5]. Stem cells have the capability of differentiating and organising to occupy the organ-specific niche. Studies have shown that organoids tend to develop in specialised macro- and microenvironments located within each tissue where stem cells reside, known as niches [6–8]. These niches are involved in the modulation and facilitate the regulation of complex signalling pathways that guide the fate of the cells [9]. Even though the origin of the stem cell is not from the surrounding tissue or organ, it finally differentiates according to the niches where it is cultured. For instance, a single neural and dental epithelial cell can be reprogrammed into mammary epithelial cells that can regenerate the mammary epithelial tree when transplanted into the mammary gland microenvironment [10, 11]. Depending on the tissue, the niche components can be derived from the cells or exogenously incorporated into the system from the ECM substrates, small signalling molecules, growth factors, and mechanical forces such as tension, rigidity, and even fluid flow [7, 12]. The complex interplay of all the components creates a spatially and temporally organised dynamic environment in a structure and function that facilitates the self-renewal/differentiation of stem cells. The self-assembly of cells in organoids nurtures and maintains tissue homeostasis, which has crucial effects on stem cell functions. The interactions between cells and ECM are important because it has been demonstrated that phenotypes can supersede genotypes by these interactions [13, 14]. Any changes in the ECM are identified by cell receptors that provide cues that eventually determine gene expressions [15–17].

The biomaterials with specified mechanical and/or biochemical properties to support cell adhesion and development are gaining attention to substitute the conventional organoid culture scaffolds which utilise natural ECM [18]. The properties of biomaterials have been shown to guide the lineage of mesenchymal stem cells [19, 20]. The mechanical microenvironment influences key cell structure and function aspects, causing cells to change their morphology, motility, proliferation, and differentiation state [21]. This chapter discusses the regulation of cell behaviours through various ways and different types of biomaterials used as matrices in organoid development.

Extracellular Matrix and Organoids

Multiple methods are used in organoid development, including embedding cells in extracellular matrix components or hydrogels [22, 23], cultivation in the spinning

bioreactor, 3D bioprinting, and microfluidic techniques [24]. In all approaches, the initial step is selecting the type of cells to be expanded, followed by cultivating the cells in a homogeneous medium with or without differentiation cues for further development [25]. Generally, the key signalling pathways to regulate developmental patterning are activated or inhibited using commercially available morphogens and signalling inhibitors. Cultures are developed in a way that enables them to expand in three dimensions, either by aggregating cells into 3D structures or by integrating cultures into a 3D matrix [26]. The patterns in stem cell activities are influenced by the external signals obtained from its local niche. These signals include soluble growth factors and hormones, cell-cell interactions, and local cues from the ECM [27, 28]. Collectively, all these variables function in tandem. As a result, the stem cells retain their conditions and perform effective regenerative activities for long-term functionality and maintenance of the cells throughout their lifetime. The most common way to encourage the 3D characteristic of organoids is to use solid ECMs that promote cell adhesion and growth.

Cell growth and differentiation are highly dependent on physical and biochemical stimuli [29, 30], enabling self-organisation in organoid culture [31]. When cultured in a 3D environment, cells are characterised by various interactions with other cells and the ECM components. The cell-matrix interaction is reciprocal signalling cascades that influence cell development. Multiple functions of ECM include its continuous interaction with cells by acting as ligands for cell receptors such as integrins, the release of growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), and other signalling molecules locally. The ECM components released from ECM cleavage also influences its architecture and cell behaviour [32]. The cells secrete ECM components that dynamically remodel the ECM, whilst both physical and biochemical properties of the ECM modulate several adhesion-related cell functions and influence the cell fate through interactions with cell-surface receptors, respectively [33, 34]. These complex processes initiate regulation of the cell behaviour, facilitate cell survival, shape, migration, proliferation, and differentiation, lead to the morphology and physiology that occur in vivo [35]. Any changes in ECM components are expected to significantly influence the biomechanical and physical properties of the ECM, leading to a disordered network and eventually a loss of organ homeostasis and function [36]. In essence, all these properties are closely interrelated and can affect each other.

The natural ECM is a complex set of proteins and proteoglycans that encompasses the cells, provide stability and regulates the signalling of the growth factor in cells [9, 37–40]. The ECM was regarded only for its structural function, stability, and support for the surrounding cells in the past. However, it is clear that it also has fundamental functions by discovering specific receptors for matrix glycoproteins, particularly by generating various complex signals that could influence cellular events at the molecular level [41]. Another main component in ECM is non-collagenous glycoproteins, which are adhesive glycoproteins consisting of various macromolecules from the ECM family that attach to cell surfaces, with several binding areas that can interact with collagens and proteoglycans [42, 43]. Fibronectin, laminin, vitronectin, thrombospondins, and tenascin are among them. In addition, some interact with integrins

and other receptors expressed on the cell surface and collagen or other ECM components [44]. Their function and differential expression encompass a broader spectrum of tissues and different cell populations in pathological and healthy environments, which are still not fully understood.

Despite the difficulties in comprehending the specifics of the cell microenvironment, biomaterials imitating the bulk ECM macroenvironment have become widely available and have been successfully employed to promote the growth of cells and organoids in vitro and in vivo. These are primarily composed of 3D polymer scaffolds and hydrogels supporting cellular adhesion and enabling nutrition delivery, biocompatibility, structural similarity to native bulk ECM, and customisable biochemical and biophysical characteristics discussed later.

The matrix microenvironment regulates cell behaviour through various ways, including cell-matrix relationships formed by cell-adhesive ligands attached to cell-surface receptors, mechanical properties such as stiffness, stress relaxation, stress stiffening, geometry, and matrix degradability, as described below.

Cell-Adhesive Ligands

The natural ECM comprises many cell-adhesive ligands, which provide sites for cell adhesion [45, 46]. The actions of cell-adhesive ligand binding cause changes in the cellular cytoskeleton leading to cell spreading [47] and migration [48]. While the active pulling actions on their surrounding environment lead to rearrangement of the ECM and clustering of cell-surface receptors, intracellular signalling sites often initiate changes in gene expression [44, 49]. All actions influence the cell behaviours such as cell motility [50], spreading [51], differentiation and angiogenesis [52]. The integration of naturally derived proteins such as fibronectin-, laminin- and collagen-derived peptides into the engineered scaffolds provides cell adhesive ligands, enhancing cell phenotype [9].

Mechanical Properties

In addition to biochemical stimuli, the mechanical properties of tissues play an important role in directing cellular behaviours [53, 54]. Different tissues comprise different ECM components and cells, with a regulated organisation that makes each organ different in response to mechanical stimuli. Based on these, organs have stiffness values that suit their biological roles.

The stiffness of ECM plays a crucial role in tissue development and pathologies because it affects multiple cellular properties, such as cell adhesion, spreading, proliferation, differentiation, and apoptosis [9, 55]. The stiffness of the tissue is transduced into biochemical signals by the cell surface receptors, which precisely interact with the same type of molecules on the neighbouring cells, such as cadherins, or

with integrins in the ECM [56]. The stiffness of tissue microenvironments in the body varies from compliant, such as in the brain and lungs, to more rigid cartilage and bones. Injuries affect the tissues' homeostatic stability, structure, and function [151, 152]. If the microenvironment is abnormally rigid, mechanical control can result in cell dysfunction. Excess ECM components accumulate in injured tissue, making it stiffer and more rigid than normal tissue, resulting in tissue dysfunction as seen in fibrotic disease, tumours, and chronic inflammation [153]. Following a heart attack, rigid scar tissue grows, resulting in an abnormally low rate of MSC differentiation following a heart transplant [154]. MSCs and other forms of stem cells proliferate, migrate to the injured area, and differentiate in response to the stiffness of the surrounding matrix [19, 155]. The stiffness of the ECM can be tailored to mimic the tissues to be reproduced, and it is highly dependent on the concentration of the main component from different polymers (both natural and synthetic) and the level of the crosslinker without affecting the cell viability [15]. Depending on the scaffold fabrication methods, the stimuli can be directly applied to the biomaterial to prompt a response that can be regulated both temporally and spatially, allowing precise control over the cellular response as needed.

Viscoelasticity of the natural ECM allows the energy of the applied stress to be dissipated through time-dependent processes, known as stress relaxation, resulting in reorganising the matrix. By comparison, in the elastic material, the energy can be 'stored', and 'recovered' during each cycle, and, hence, the stress remains constant over time [57]. It is reported that MSCs cultivated in 3D cultures are sensitive to stress relaxation rates, which demonstrated increased distribution, proliferation and osteogenic differentiation in fast-relaxing 3D hydrogels relative to slower relaxing matrices, irrespective of material stiffness [51, 58].

Stress stiffening is another important parameter that governs stem cell fate in a 3D microenvironment [59]. The natural fibrous ECM exhibit a stress-stiffening behaviour, which means that they become stiffer if the stress added reaches the critical stress value. The matrix cannot undergo further structural rearrangement at the critical stress value, and the extra stress is dispersed within the fibrous matrix [25]. Even though most synthetic hydrogels are not stress-stiffened in nature, stress stiffening matrix can be reconstructed with specific stiffness and bundling characteristics such as in helical oligo(ethylene)glycol polyisocyanopeptide hydrogels [60–62]. In addition, synthetic 3D matrices can precisely redirect the cell fate of human MSCs from adipogenesis to osteogenesis simply by altering the polymer chain length whilst maintaining the matrix stiffness and ligand density [59]

Matrix Geometry

The porosity, pore sizes, and interconnectivity affect the functionality of the matrix. High porosity, open and interconnected pores, which also are directly influenced by the pore size, are necessary to enable cell infiltration or proliferation [63]. In addition, tissue vascularisation and new tissue development can be significantly faster [64].

The native ECM consists of a fibrous network with pore sizes approximately equal to the size of cells, allowing nutrient transport and cell migration and growth [9]. The architecture can be built and manufactured in organoid development, using physical and chemical cross-link techniques to obtain the most appropriate biomaterials for a particular tissue formation [65]. Different forms of 3D cell culture matrices can be classified according to their geometric design. Naturally derived fibrous matrices, such as ECM and hydrogels, are made up of fibrous proteins, with pores sizes almost equal to the size of a cell. The distance between cross-linked matrix determines the mesh size and creates empty volumes that are usually much smaller than cells, present in many cross-linked hydrogels with a mesh-like structure [25]. This tight mesh will prohibit cell proliferation and movement in the absence of degradation or other types of material remodelling [66].

The orientation and diameter of the fibres within the material determines the behaviour of the cells. For example, fibre alignment facilitates the selection of fate in human tendon stem cells compared to randomly aligned fibres [67]. However, MSCs are oriented more strongly towards the fate of the tendon cells in fibre-diameter relative to fibre alignment than tendon stem cells [68]. However, these variations may also be caused by differences in cell type or material chemistry.

Matrix Degradation and Remodelling

The constant remodelling of natural ECM is regulated by the cell-mediated processes of degradation and production of ECM components. The degradation rates of the biomaterial have a major effect on cell activity. In general, biomaterial degradation can be either cell-mediated or cell-independent [25]. Cell-mediated degradation is facilitated through enzymes, such as proteolytic enzymes, which degrade the matrix by cleaving specific amino acid sequences. In contrast, cell-independent material degradation is caused by hydrolysis of chemical bonds and disruption of physical cross-links within and between the material, respectively. For instance, the alginate biopolymer is ionically cross-linked by calcium ions, and by removing the calcium, the alginate matrix can be disrupted and degraded [69, 70]. The ECM-digesting enzymes produced by the cells facilitate the rearrangement of the microenvironment by expressing new polymers [71]. Biomaterials can be designed to provide a matrix that allows degradation and remodelling at a rate desirable for embedded cells. Table 8.1 summarises the various ways of matrix microenvironment regulates cell behaviours.

Biomaterials as Matrices in Organoid Development

The discovery that natural ECM can be transformed into hydrogels significantly improved their in vitro and in *vivo* applications. Hydrogels are highly hydrated

Table 8.1 Summary regulation of cell behaviours through matrix microenvironment

Characteristic of matrix	Effects on cells
Cell surface ligands	– Change the cellular cytoskeleton leading to cell spreading and migration – Active pulling actions on their surrounding environment lead to rearrangement of the ECM and clustering of cell-surface receptors, initiate changes in gene expression in the nucleus
Stiffness	– Stiffness of the tissue is transduced into biochemical signals by the cell surface receptors – Affects multiple cellular properties, such as cell adhesion, spreading, proliferation, differentiation, and apoptosis
Stress relaxation	– Allows the energy of the applied stress to be dissipated through time-dependent processes resulting in the reorganisation of the matrix
Stress stiffening	– Fibrous ECM becomes stiffer when additional stress reaches the critical stress value, restraining the matrix from undergoing further structural rearrangement at the critical stress value – Can precisely redirect the cell fate
Geometry	– Porosity, open and interconnected pores, directly influenced by the pore size, allows cell infiltration or proliferation – The orientation and diameter of the fibres within the material determines the behaviour of the cells
Matrix degradation	– Cell-mediated degradation is facilitated through enzymes, such as proteolytic enzymes, which degrade the matrix by cleaving specific amino acid sequences – The cell-independent material degradation is caused by hydrolysis of chemical bonds and disruption of physical cross-links

polymer materials that contain more than 30% water by weight and maintain structural integrity through physical and chemical cross-linking of polymer chains [23]. These polymer chains are biomaterials that are extensively investigated as extracellular matrices to mimic the natural complex and functional microenvironment of native stem-cell niches, which can be classified into naturally derived, synthetic or hybrid composites of both [72]. Furthermore, the ability to customise the biochemical and physical parameters such as mechanical properties or permeability in the microenvironment of the cells is a significant benefit of using synthetic biomaterials in tissue engineering [73]. Some biomaterials which are commonly used in organoid development are listed below.

Natural ECM

a. Decellularized ECM and hydrogels

Decellularisation is the process of physical, chemical, and enzymatic removal of cellular components and antigens while maintaining the essential structural and functional ECM proteins and the natural structure of glycosaminoglycans. As a result, a natural, biocompatible structure is free of the possibility of adverse effects at the graft site, such as inflammatory reaction and immunological rejection. However, the process can damage ECM proteins, demanding a balance between sufficient cell removal and ECM integrity preservation. The ECM can be processed into hydrogels in a variety of ways once it has been decellularised. The solubilisation of ECM proteins with acids and enzymes is the core concept of hydrogel formation. Pepsin is widely applied for this, as it cleaves the nonhelical protein regions outside of collagen's triple helix protein structure, which is responsible for intramolecular connections between collagen fibrils, producing monomeric components. Pepsin is inactivated to physiological pH following neutralisation, and the hydrogel is formed via a collagen-based self-assembly technique at 37 °C or below [74, 75].

Evidence from preclinical studies and experimental human transplantation has demonstrated effective in vivo tissue regeneration, suggesting that decellularised ECM provides not only structural support but also biochemical signals that are necessary for tissue regeneration, including ECM tissue-specific proteins, as well as soluble factors absorbed inside the ECM protein network [74–79].

Matrigel, a natural-derived ECM purified from Engelbreth-Holm-Swarm (EHS), is a reconstituted basement membrane harvested from mouse sarcoma is the predominantly used matrices to develop organoid [31]. The EHS matrix is incorporated with many different components of the natural ECM, such as laminin, collagen IV, and entactin [80, 81]. In addition, biological factors including basic fibroblast growth factor, epidermal growth factor (EGF), insulin-like growth factor 1, transforming growth factor-beta, platelet-derived growth factor, and nerve growth factor are also added, which give a dynamic environment for embedded cells to facilitate cell attachment and can be degraded and remodelled by organoid enzymes [82]. A few successful examples of organoid development include intestinal [83], cerebral [84, 85], liver [86] and mammary gland organoids. Nevertheless, the use of Matrigel has been reported to have several drawbacks. Compared to normal tissues, which typically have a stiffness of ~100–100,000 Pa, EHS matrices are soft materials with stiffnesses of ~20–450 Pa [87, 88], and the physical properties cannot be modified. Furthermore, the composition of EHS are poorly defined, incompatible with good manufacturing practices (GMP), and display batch-to-batch heterogeneity, which hinders the production of robust processes [31, 72]. Furthermore, the translational potential of organoid cultured in EHS-matrix into clinical applications are limited due to the mouse tumour origin of the EHS [89].

b. **Other naturally derived biomaterials**

Other proteins and polysaccharides were studied to produce new functional tissues and even more efficient models for organoid cultures among natural biomaterials. The single-component protein-based hydrogel matrices such as collagen Type 1 were compared with Matrigel in intestinal organoid development. According to Jabaji et al. [90], Type I collagen hydrogel generated from healthy intestinal crypts can serve as a well-defined extracellular matrix for repeatable and long-term in vitro maintenance and expansion of fully elaborated human intestinal epithelium. Furthermore, the collagen-based matrices produced both classic in vitro epithelial structures (known as enteroids) and a new sheet-like growth pattern that was not observed in Matrigel. In another study by Jee et al. [148] using type 1 collagen, Ham's F12 nutrient mixture, and bicarbonate showed that mouse small intestine–derived organoids, stomach-derived organoids, and human colon–derived organoids were successfully grown in the collagen-based matrix and had similar properties compared to those cultured in Matrigel. Among other proteins, the fibrin-based hydrogel has also been investigated as matrices for organoid development with collagen IV, heparin, and laminin supplementation. Apart from physical support, the naturally occurring Arg-Gly-Asp (RGD) adhesion domains on the scaffold, supplementation with laminin, are key parameters required for murine and human epithelial organoids formation and expansion [91].

Gelatin, derived from collagen hydrolysis, provides suitable chemical and biological cues for hosting various cells [92]. Due to its chemical similarities to the ECM in native tissues, the biocompatibility, biodegradability, low antigenicity, cost-effectiveness, abundance, and available functional groups that allow chemical modifications with other biomaterials or biomolecules, gelatin is a promising material as a scaffold with therapeutic and regenerative properties [93]. However, gelatin is limited by its poor mechanical properties, fast enzymatic degradation, and low solubility in concentrated aqueous media [94, 95]. Therefore, gelatin is commonly cross-linked with other natural or synthetic biomaterials to enhance its properties. For example, gelatin methacryloyl (GelMA), a commercially available hybrid hydrogel matrix, undergoes photoinitiated radical polymerisation and contains most methacrylamide groups and a minority of methacrylate groups [96, 97]. Finely tuned mechanical properties of GelMa can be generated by adjusting the proportion of methacryloyl substitution, providing its application in organoids and as bioink in 3D bioprinting technology [98–100]. In addition, gelatin has been combined with other biomaterials such as polysaccharides or synthetic polymers such as polycaprolactone (PCL) [101], poly(lactic-co-glycolic acid) (PLGA) [102], and poly(L-lactic acid) (PLLA) [103] to enhance complex hybrid polymeric frameworks [93]. It was observed that fibroblasts were encapsulated in gelatin hydrogels for over 28 days, resulting in substantial cytoplasmic spreading and the development of cellular networks. When gelatin hydrogels were cross-linked with other polymers such as poly(ethylene glycol) diacrylate (PEGdA), the encapsulated fibroblast showed more extensive cytoplasmic spreading and the formation of cellular networks over 28 days [104].

Polysaccharide-based materials include chitosan and hyaluronic acid (HA) [105] and alginate [106, 107], which are derived either from animals, plants, or microorganisms. The composition of these natural materials allows many molecular interactions between receptors expressed on the cell surface and their neighbouring counterparts and the ECM, which results in cell adhesion, proliferation, and subsequent differentiation [32, 43, 108, 109]. The complex interactions play essential roles to provide an adhesive and structural substrate to which integrins and other adhesive cell receptors can bind, subsequently involved in the activation and regulation of pro-survival signalling cascades. In addition, some bioresponsive molecules may provide signals that modulate cell adhesion and cell differentiation and cell growth by cell-cell and cell-ECM interactions, either tethered or insoluble form [110].

Chitosan is a linear polysaccharide derived from partial deacetylation of chitins of crustaceans which has several distinct physical and chemical properties from its amino and hydroxyl groups. These reactive groups contribute to the flexibility and ease of functionalisation [111]. The structural and functional similarities of chitosan with glycosaminoglycans (GAGs) are present in native ECM. Thus, chitosan is biocompatible and used predominantly in cartilage engineering [112, 113]. Chitosan hydrogel has proven to be an attractive biomaterial in tissue engineering and regenerative medicine due to its injectability, enzymatic degradability, and high biocompatibility [114, 115]. Apart from promoting adipose-derived stem cells (ADSC) engraftment, survival, and homing by mediating chemokine recruitment and ROS scavenging, the hydrogel also promotes cardiac differentiation of brown adipose-derived stem cells (BADSCs) by enhancing collagen production [116].

Hyaluronic acid (HA), commonly referred to as hyaluronan, is a linear polysaccharide composed of alternating α-1,4-D-glucuronic acid and β-1,3-N-acetyl-D-glucosamine disaccharides, connected by β(13) bonds [117]. Structurally, HA carries a negative charge, and it is a significant macromolecular part of the intercellular matrix of most connective tissues, such as cartilage, human eye vitreous, umbilical cord and synovial fluid [118]. Because of its abundant hydrophilic groups, such as hydroxyl, carboxyl, and acetamido groups, HA can be readily formed into hydrogels by forming hydrogen bonds. By inserting methacrylate groups into the HA backbone via the hydroxyl groups, a photocross-linkable methacrylated HA (MeHA) hydrogel can be produced. HA can promote cell differentiation, proliferation, and matrix secretion [119] and has been documented to provide an effective niche for stem cells to differentiate into chondrogenic lineages [120–123]. It was reported that MSCs derived from human adipose tissue and placenta formed 3D spheroids on the chitosan membrane. When the chitosan-HA membrane was used, the spheroid formation was faster and larger than chitosan alone [105].

Alginate is an anionic polymer from the family of naturally occurring polysaccharides extracted from brown seaweeds. Although it has no inherent instructive cell properties, alginate promotes human intestinal organoids growth and leads to epithelial differentiation that is virtually indistinguishable from Matrigel. Moreover, when transplanted in vivo, alginate-grown HIOs mature to a similar degree as Matrigel-grown organoids, both of which resemble human foetal intestines. The alginate matrix can support in vitro development of human iPSC-derived intestinal organoids for at

least 90 days, suggesting that mechanical support is sufficient for such organoid cultures in the absence of other ECM signals. This is possible because organoids create their niche within the culture [106].

Furthermore, alginate can be functionalised with an RGD (Arg-Gly-Asp) peptide sequence present in major ECM proteins, such as fibronectin, through its functional amino acid and surface charge to enable ECM interactions and signalling [18]. Functionalisation of alginate beads with collagen type 1 and transforming growth factor-β1, cultured with human lung fibroblast and iPSC-based mesenchymal cells in a rotating bioreactor, has resulted in the formation of a close-packed structure which restricts cellular attachment and proliferation to the interstitial space between the beads. Alternatively, it causes the formation of acellular regions within the culture, mimicking the alveolar architecture of lungs used to model idiopathic pulmonary fibrosis [124].

Engineered matrices are a promising alternative to conventional organoid culture scaffolds because they provide a great tunability, are fully chemically defined, and can be easily manufactured using standard techniques, which can mimic key features of natural ECMs.

Synthetic Matrices

Due to the limitations encountered by the natural matrices, efforts have also been demonstrated to construct artificial matrices that can solve these challenges in clinical translation, such as in mouse [125] and human [126] organoids. Furthermore, synthetic matrices can be designed and modified by covalently cross-linked with other biomaterials to form a chain polymerisation of macromers to optimise their physical and biological properties.

Poly(ethylene glycol) (PEG) is one of the most widely used synthetic biomaterials in organoid development. PEG has a high solubility in aqueous media, biocompatibility and strong resistance, and tunable mechanical and chemical properties, particularly biomedical applications [127]. In addition, PEG-conjugated medications have been licensed for safe use in humans by the U.S. Food and Drugs Administration (FDA) [128–131]. The PEG macromer's end hydroxyl groups can be functionalised with reactive end groups to allow for a range of cross-linking chemistries such as vinyl sulfone, acrylate, amine, and maleimide end groups [132, 133]. Additionally, biological features such as integrin-binding peptides can be added to PEG macromers to produce a microenvironment that encourages cell adhesion [132]. Biodegradable PEG hydrogels can also be produced by copolymerisation with other polymers such as PLA, PLGA, poly (propylene fumarate), and other naturally occurring biomaterials hyaluronic acid, fibrinogen and chitosan. PEG-macromers functionalised with several types of binding peptides were reported to support the human intestinal enteroids and endometrial organoids [134], and the engraftment of human pluripotent stem cell (hPSC)-derived organoids (HOs) and further accelerated colonic wound

repair [135]. A cross-linked transglutaminase (TG-PEG/HA) hybrid hydrogels developed by [136] could maintain, expand, or differentiate human bone marrow-derived stromal cells and human hematopoietic stem and progenitor cells h which had superior properties concerning material handling, structural stability and reducing in vivo macrophage penetration relative to pure PEG or pure HA.

Poly(lactide-co-glycolide) or poly(lactic-co-glycolic acid) (PLGA) is a linear aliphatic copolymer synthesised at different proportions between its constituent monomers, lactic acid (LA) and glycolic acid (GA). PLGA can be hydrolytically degraded in the body to produce both endogenous monomers and physiologically metabolised through the Krebs cycle. Its hydrophobic polymer can be identified by the reticuloendothelial system and eliminated by the liver or spleen through phagocytosis [137–139]. The use of PLGA is consistent with low toxicity. Therefore it has become one of the most desirable biomaterials used as a drug delivery carrier, sutures, and other tissue engineering applications in humans and licensed by the U.S. FDA and European Medicine Agency (EMA) [140, 141].

Meanwhile, polycaprolactone (PCL) is an aliphatic polyester polymer consisting of hexanoate repeat units. Due to the low melting temperature, exceptional blending compatibility, hydrophobicity, and easily handled, it becomes a chosen polymer mainly for biomedical applications [142]. For example, PCL was used to prepare long-term implants due to its substantially slow degradation rate. In addition, its scaffold can mimic the extracellular matrix of natural substances, thus supporting the three-dimensional (3D) cell culture in tissue engineering and regenerative medicine [143].

PLGA fibre microfilaments have been used in brain organoid and showed enhancement of neuroectoderm formation and improved cortical growth [144]. In addition, transplanted microporous PLGA scaffold seeded with human lung organoids (HLO) indicated enhanced epithelial structure and organisation cellular similar to the native adult human lung [145].

Three microporous polymer scaffolds, PLGA, PEG, and PCL, were used to study the various stages of HLO development in immunocompromised mice. Both PLGA- and PCL-transplanted HLO exhibited more immature lung progenitors, while PEG scaffolds demonstrated slowed development and maturation [146]. In addition, when compared to the conventional soluble group, hybrid PLGA scaffolds seeded with human pluripotent stem cell-derived hepatocyte-like cells with additional growth factors showed similar expression levels of hepatocytic markers, ultrastructure, and functional characteristics [147]. Table 8.2 summarises the type of matrices used in organoid development.

The inability of synthetic polymers to provide the biochemical signals required to "communicate" with the cell is the key drawback of using them as building blocks for ECM mimics. However, by incorporating signalling biomolecules, synthetic polymers can be functionalised to resolve this limitation (Fig. 8.1).

Table 8.2 Types of matrices used in organoid development

Cells sources	Matrix	Nature of matrix	References
Porcine decellularised small intestine submucosa	Decellularized ECM	Natural	Hirota et al. [74]
Porcine decellularised small intestine mucosa/submucosa	Decellularized ECM	Natural	Giobbe et al. [23]
Gastrointestinal	Collagen type 1	Natural	Jee et al. [148]
Porcine gastrointestinal	Collagen type 1	Natural	Jabaji et al. [90]
Human adipose and placenta stem cells	Chitosan and chitosan-hyaluronan	Natural	Huang et al. [105]
Intestinal	Alginate	Natural	Gjorevski et al. [125]
Intestinal	Alginate	Natural	Capeling et al. [106]
Epithelial organoids	Fibrin/laminin	Natural	Broguiere et al. [91]
Bone marrow	TG-PEG/HA	Hybrid	Vallmajo-Martin et al. [136]
Human lung	PLGA	Hybrid	Dye et al. [145]
Porcine hepatic islet	PLGA microspheres	Hybrid	Gibly et al. [149]
Brain	PLGA	Synthetic	Lancaster et al. [144]
Human intestinal and endometrial	PEG—8 arms	Synthetic	Hernandez-Gordillo et al. [134]
Pancreatic progenitor cells	Matrigel	Synthetic/semi-synthetic	Greggio et al. [150]
Intestinal	PEG 4 arm	Synthetic/semi-synthetic	Cruz-Acuña et al. [135]

Recommendations and Suggestions

Organoids derived from human pluripotent stem cells provide a reliable tool for a wide range of disease modelling and a potential drug screening and toxicity testing strategy. Controlling stem cell fate to promote proliferation and differentiation into specific cell types, on the other hand, remains a challenge. The stem cell fate is determined by the source of the cells or tissues, the protocol, growth factors and morphogens used, the media composition used to maintain the intended organ's structure and functional characteristics, and finally, the appropriate matrices required for multiple stages of organoid development and subsequent in vivo propagation. One of the most difficult aspects is designing a scaffold that accurately replicates the spatial presentation of signals to cells. Conventional 3D cultures are flooded with biochemical signals with no spatiotemporal control, resulting in significant discrepancies in organogenesis in vivo and in vitro. Therefore, it is critical to integrate

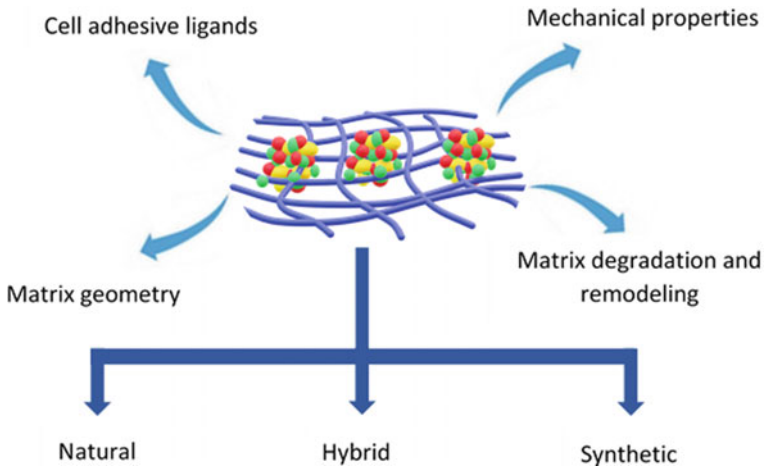

Fig. 8.1 Regulation of cell behaviours through matrix microenvironment and types of matrices used in organoid development

niche components for in vitro culture that drive organoid formation into a 3D culture scaffold capable of simulating in vivo cell-ECM interactions, as a single type of matrix would be incapable of supporting organoid development over an extended period of time. Alternatively, a niche-specific matrix can be created by decellularising patient-derived tissues or cells to match the unique design of the respective organ. It can be further cross-linked with customisable synthetic matrix structures to enhance the characteristics and facilitate organoid development when transplanted in vivo. Indeed, extensive studies have been undertaken throughout the world to address these limitations.

Conclusion

Organoids offer the potential to study human physiology in vitro, but the maturation of organoid cultures depends on the ability of the stem cells to create highly ordered structures. Numerous material properties, including the presentation of cell-binding ligands, matrix dynamics, structural geometry, and degradability, influence stem cell development and key design parameters for organoid matrices. 3D ECMs can be designed to fine-tune the various structural and biochemical parameters that affect cell fate. Good comprehension of the presence of ECMs in various tissues, pathologies, and even different individuals, together with the ability to manufacture extremely complex 3D biomaterials, can enhance the development of artificial organs and the possibility to exclude the use of animal models.

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Chapter 9
Genome Editing in Organoid to Improve Understanding of Human Disease

Binhui Zhou and Yinming Liang

Abstract **Introduction**: The CRISPR/Cas9 system has been widely used in genome editing. In terms of application, CRISPR-based genome editing exhibits more advantages than ZFN and TALEN. Although delivering the CRISPR/Cas9 system is still a huge challenge, genome editing is feasible in both cell lines and organoids. The CRISPR/Cas9 system is a useful and effective tool for studying the mechanisms of single-gene or multi-gene diseases and developing genetic models to find new therapeutic targets. In this chapter, we focus on the application of the CRISPR/Cas9 system in various types of organoids and outline the effects of using this technology. **Methods**: The databases PubMed and Google were used to select literature with keywords genome editing, CRISPR/Cas9 system and neurosphere organoids. **Results**: Organoids provide a reliable tool for extensive disease modelling and therapeutic effect evaluation after CRISPR-based gene editing. After the CRISPR/Cas9 system performed gene editing on organoids of many diseases, the unhealthy phenotype of organoids has been greatly improved. However, the off-target effect of Cas9 and the method of delivering sgRNA to target cells are problems to be solved urgently. **Conclusion**: The CRISPR/Cas9 system has exhibited unparalleled gene editing effects. It performs gene editing on mutated, deleted or overactivated genes in organoids of various disease types, showing a very promising clinical treatment prospect. As long as the off-target effect of Cas9 can be controlled and an effective delivery method can be developed, CRISPR/Cas9 system will play a huge role in the clinical treatment of various diseases.

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Keywords Genome editing · CRISPR/Cas9 system · Neurosphere organoids

Abbreviations

CRISPR	Clustered regularly interspaced short palindromic repeats
sgRNA	Small guide RNA
HSPC	Hematopoietic stem and progenitor cells
DNA	Deoxyribonucleic acid
mTORC1	Mammalian target of rapamycin complex 1
NFAT1	Nuclear factor of activated T cells
NRF2	Nuclear factor erythroid 2-related factor 2
HIV	Human immuno-deficiency virus
CAR-T	Chimeric antigen receptor T-cell
RPE	Retinal pigment epithelium
ssODN	Single-stranded donor oligonucleotides
CCR5	C-C chemokine receptor type 5
XLRP	X-linked retinitis pigmentosa
ONL	Outer nuclear layer
WT	Wild type
VEGF-A	Vascular endothelial growth factor A
Hif-1α	Hypoxia-inducible factor-1 alpha
RNP	Ribonucleoprotein
LDL-C	Low-density lipoprotein cholesterol
bp	Base pair
LQTS	Long QT syndrome
EGFR	Epidermal growth factor receptor
NSCLC	Non-small cell lung cancer
EMT	Epithelial-to-mesenchymal transition

CRISPR/Cas9 Technology Used in Neurosphere Organoids

Neural stem cells are central primordial cells that can self-renew and differentiate in multiple directions [1]. Adult animals' embryonic tissues and subventricular zone, as well as the hippocampus dentate gyrus, are the most common sources [2]. A neurosphere is a type of neural stem cell grown in vitro in a mitogen-containing tissue culture media. The cells in the suspended spherical cell cluster are thought to maintain neural stem cells' basic proliferation and differentiation capabilities. After removing the mitogen, the created primary neurospheres can be distributed and passed many times, and the most essential thing is that they can differentiate into neurons and glial cells [1]. Reynolds et al. published the first method for producing and culturing

neurospheres using mouse striatal neural stem cells in 1992 [3]. Because of the bidirectional advantages of numerous passages and inducible differentiation, this approach is commonly utilized in the in vitro cultivation of neural stem cells. Neurospheres are also utilized as organoids to research neuropsychiatric illnesses, such as epilepsy [4, 5], medulloblastoma [6], primary microcephaly [7], neuroblastoma [8] and especially glioblastoma [9], [10–14].

Unprovoked seizures are a symptom of epilepsy, a spectrum of neurological illnesses. Among them, focal epilepsy caused by environmental and genetic factors is the most frequent kind of epilepsy. Dibbens et al. showed that DEPDC5 (DEP domain-containing 5 protein) gene defect promotes autosomal dominant focal epilepsies, with insufficient penetrance and varied expressivity [4]. And Hughes et al. discovered mTORC1 hyperactivation in fetal brain lysates, nutrient-depleted neurospheres, and mouse embryonic fibroblasts [5]. Therefore, using CRISPR/Cas9 technology to restore mTORC1 to its normal activity level in the heterozygous DEPDC5 mutant may be a treatment for epilepsy.

Medulloblastoma is the most frequent malignant juvenile brain tumor that develops from the cerebellum. Sonic Hedgehog, Wingless, Group 3 and 4 are the four major molecular subgroups of medulloblastoma [6]. High MYC expression due to its amplification in approximately 15–20% of cases characterizes the Group 3 subgroup, which accounts for around 25% of all medulloblastoma cases [15]. Vo BHT et al. created CRISPR-Myc, a Group 3 medulloblastoma cell model, by using CRISPR/dCas9 system with combinatorial sgRNAs to impose the expression of Myc in Trp53-null neurosphere cells that transplanted into naive mice brain, and discovered that JQ1, a BET inhibitor, reduced the development of neurospheres in CRISPR-Myc cells by suppressing MYC expression [6].

Primary microcephaly is a congenital brain disorder marked by a head size significantly smaller than standard deviations below the age and sex mean, resulting in mild to severe mental deficits and a shorter lifespan. Using exome sequencing, DiStasio et al. discovered a mutation that is autosomal recessive causing an amino acid change in the WD40 domain of COPB2 in two infants with primary microcephaly. They used CRISPR/Cas9 technology to create an allelic sequence in the mouse to better investigate the significance of Copb2 in brain development. Copb2 is required for the early phases of embryogenesis, according to two independent null alleles. The phenotype of mice homozygous mutation ($Copb2^{R254C/R254C}$) appears to be identical to that of humans, owing to changes in corticogenesis. Mice exhibiting a severe phenotype, such as low birth weight, augmented brain apoptosis, and death during the first seven days of life. Immunohistochemistry of the $Copb2^{R254C/Zfn}$ brain indicated a decrease in layer V ($CTIP2^{+}$) neurons, despite the cortex's overall cell density remaining intact. Furthermore, neurospheres produced from animals with Copb2 mutations expanded at a slower rate than control neurospheres [7].

One of the most dangerous solid malignancies of childhood is neuroblastoma (NB), which develops from neural crest-derived sympathoadrenal progenitors [16, 17]. Flahaut et al. found that ALDH1A3 (acetaldehyde dehydrogenase family 1 member A3) was widely expressed in NB cell lines and correlatedwith poor survival

and high-risk prognostic factors, and specific knockout of ALDH1A3 decreased neuroblastoma cell clonogenicity and tumour-initiating cell [8].

In the adult central nervous system, the most frequent malignant neuroepithelial tumor is glioblastoma multiforme (GBM) [18]. Patients with this tumor have a survival duration of no more than 12 months due to its aggressive growth and great heterogeneity [19]. Researchers have discovered that a variety of protein molecules are involved in the progression of GBM. For example, Ranjan and Srivastava discovered that utilizing CRISPR/Cas9 to block the GLI Family Zinc Finger 1 (GLI1) gene resulted in improved GBM cell growth-suppressive effects of penfluridol, an antipsychotic medication [9]. Furthermore, Han et al. discovered that knocking down the Quaking homolog (QKI) boosted GLI1 mRNA levels, resulting in the maintenance of glioblastoma stem cell stemness and an increase in GBM cell invasiveness [10]. Furthermore, Ali et al. showed that knocking out Ataxia-telangiectasia mutated (ATM) in glioblastoma cell lines (LN18 and LN229) in the absence of p85α hampered cancer cell motility and invasion, inhibited three-dimensional-neurosphere formation, and enhanced chemotherapeutic toxicity to cisplatin [13]. Similarly, Thakur et al. discovered that knockout of SAT1 in primary glioblastoma lines by using the CRISPR/Cas9 system resulted in a substantial inhibition of neurosphere formation [14], and Jiang et al. found that NFAT1 suppression via CRISPR/Cas9 reduced the survival, invasion, and self-renewal of glioma stem-like cells in vitro and prevented tumorigenesis in vivo [11]. Furthermore, Godoy et al. discovered that knocking down the NRF2 gene with CRISPR/Cas9 in U87MG cells lead to reduced neurosphere self-renewal, increased differentiated cells, and inhibited proliferative potential after gamma ray irradiation [12].

CRISPR/Cas9 Approach Used in the Hematopoietic System

The hematopoietic system contains the blood's produced components, as well as the lymph nodes, spleen, bone marrow, and reticuloendothelial tissue, all of which are specifically designed to supply the body with a large potential for cell regeneration. Hundreds of millions of people worldwide suffer from hematologic diseases, including blood and blood-forming organ problems. In addition to blood cell malignancies, hematologic diseases include rare genetic abnormalities, anemia, thrombocytopenia, HIV-related ailments, sickle cell disease, chronic granulomatous disease, X-Linked agammaglobulinemia, etc. The majority of these diseases are genetic conditions caused by a single gene. In recent years, CRISPR/Cas9 has been reported to be widely utilized in the clinical treatment of hematopoietic illnesses as a result of numerous studies showing its great efficiency in single gene editing.

Acute myeloid leukemia (AML) is a life-threatening blood malignancy. CD33 has long been a focus for AML immunotherapy. However, CD33 expression on normal hematopoietic cells has been shown to cause "on-target, off-leukaemia" toxicity, resulting in substantial morbidity/mortality from deep cytopenia, restricting the use of CD33-directed immunotherapies. As a result, eliminating CD33 from resting

HSPC will be an excellent way to create a hematopoietic system that is resistant to CD33-targeted treatment and will allow CAR-T cells to be used to selectively target AML. For this reason, Miriam Y Kim et al. created CD33 knockout human HSPC by using the CRISPR/Cas9 method, and found that the $CD33^{-/-}$ HSPC cells exhibited normal myeloid activity in autologous transplanted rhesus macaques. Most crucially, $CD33^{-/-}$ cells were unaffected by CAR-T cells that targeted CD33, allowing for effective leukemia therapy and without the risk of toxicity [20].

Beta thalassemia is a genetic illness marked by a lack of β-chain synthesis. When β-globin synthesis is impaired, the α-/β-chain ratio becomes imbalanced, which in turn leads to red blood cell (RBC) lysis. SOX6 is a transcription factor that plays a gene switch role in regulating γ- to β-globin. Laleh Shariati et al. discovered that using CRISPR/Cas9 system to mutate SOX6 gene binding region results in γ-globin reactivation in K562 cells [21]. In HbE/β-thalassemia, β0 allele causes no β-globin chain to form, while βE allele generate a HbE globin chain due to a nucleotide change of GAG to AAG. Based on this, researchers employed the CRISPR/Cas9 technique to successfully correct the mutation in iPSCs produced from a HbE/β-thalassemia patient, resulting in corrected iPSCs that are a β-thalassemia heterozygote. In the future, the corrected iPSCs can be artificially induced into hematopoietic stem cells and used in patient autologous transplantation to treat thalassemia [22].

The HIV causes AIDS, which is a chronic, potentially fatal illness. To date, there were approximately 40 million people across the globe with HIV/AIDS in 2021. Vaccines and drugs currently used to treat AIDS mainly target viral proteins or block the interaction between the host and the virus. However, HIV can integrate its genome into the immune cells to establish latent infection, posing a daunting challenge in the development of vaccines. Based on this, Liao et al. revised their approach to developing AIDS medicines. They modified the CRISPR/Cas9 system for intracellular defense against viruses and foreign DNA in human cells. As a result, the integrated viral genomes were destroyed in human cells, then provides long-term adaptive resistance against newly infected viruses, their expression, and replication. Furthermore, HIV-targeted CRISPR/Cas9-expressing human induced pluripotent stem cells can be induced into HIV reservoir cells and maintain resistance to HIV-1 infection [23]. Other studies have shown that the chemokine receptor CCR5 plays a key role in HIV entering human hematopoietic cells, and homozygous CCR5 mutation greatly enhances HIV-1 resistance [24, 25]. In this case, Xu et al. found that the CCR5 knockout efficiency was sustained in transplanted hematopoietic cells using CRISPR/Cas9 method. More crucially, the considerable decrease in virus concentration and increase in the number of $CD4^+$ T cells revealed an HIV-1 tolerance effect [26].

X-linked chronic granulomatous disease (X-CGD) is a primary immunodeficiency disorder caused by the inability of phagocytes to clear invading fungi and bacteria. Mutations in the CYBB gene can cause X-CGD. The CYBB gene encodes gp91phox, which is the catalytic center of NADPH oxidase 2 (NOX2). NOX2 is a phagocytic protein that can promote the production of superoxide anions, and play a role in immunoregulatory and microbicidal [27]. Patients with X-CGD are at risk of developing life-threatening infections and require antibiotic medication as a preventative

measure. The proportion of NOX2 activity in X-CGD patients' neutrophils predicts patient survival, implying that minor changes in NOX2 function could have clinical implications [27]. By using CRISPR/Cas9 technique, De Ravin et al. corrected the CYBB gene mutation of $CD34^+$ HSPCs from X-CGD patients. Interestingly, transplanting gene-edited cells into NOD SCID $gc^{-/-}$ mice can successfully generate functionally mature human lymphoid and myeloid cells. Furthermore, after gene correction, whole-exome sequencing revealed that only CYBB was gene-edited [28].

CRISPR/Cas9 Technology Used in Retinal Disorders

There are approximately 285 million visually impaired people worldwide, with 39 million of them being blind. In the developing world, photoreceptor malfunction and/or loss accounts for more than half of all cases of visual impairment. Photoreceptor is a kind of neuron with special function which plays a key role in the initial process of converting light into vision. The most prevalent kinds of retinal degeneration include inherited retinal illnesses like Leber congenital amaurosis (LCA) and retinitis pigmentosa (RP), as well as more complicated and heterogeneous retinal disorders like age-related macular degeneration (AMD) [29].

The CRISPR/Cas9 technology has been widely used in the treatment of retinal diseases in previous studies. RP is a hereditary retinopathy that is irreversible. Mutations in RPGR (also known as XLRP3) have the highest probability of causing this disease, notwithstanding its genetic heterogeneity. Deng et al. employed the CRISPR/Cas9 technology to correct the RPGR mutation, which repaired photoreceptor structure and electrophysiological characteristics, rectified ciliopathy, and restored gene expression to a level that was under control [30].

On the other hand, Arno et al. observed biallelic mutations in REEP6 in human with autosomal-recessive RP. Reep6 variant knock-in mice models created using CRISPR-Cas9 gene editing displayed clinical symptoms similar to RP, including progressive photoreceptor degradation and rod photoreceptor malfunction [31]. RP2 mutations have also been related to a severe type of XLRP [32]. The RP2 XLRP animal models, on the other hand, do not replicate this severe phenotype. As a human retinal illness model, Lane et al. developed 3D retinal organoids using gene-edited isogenic $RP2^{-/-}$ iPSCs and RP2 patient-derived iPSCs. Rod photoreceptor cells loss peaked at day 150 in both the $RP2^{-/-}$ and RP2 patient-derived organoids, followed by a weakening of the organoid outer nuclear layer by day 180. Using CRISPR/Cas9 technology to express RP2 in $RP2^{-/-}$ organoids can reverse its degenerative phenotype [33].

Autosomal recessive mutations in a variety of genes, such as RPE65, can cause LCA, which is a frequent cause of childhood-onset blindness. There is a mouse model of human LCA called Rd12, Jo et al. repaired a disease-associated nonsense mutation in Rpe65 by using CRISPR/Cas9. They discovered that when exposed to bright stimuli, the a and b waves of the electroretinograms can be restored to the level of approximately 21 and 40% of the WT mice after 7 months of dark adaptation [34].

In persons 50 years of age and older, the major cause of permanent blindness is AMD. Choroidal neovascularization (CNV) is a prominent hallmark of wet-AMD, and it is predominantly induced by angiogenic cytokines including VEGF-A, leading to further damage to the function and structure of retinal. In an AMD mouse model, Kim et al. suppressed the expression level of Vegf-A and Hif-1α by using CRISPR/Cas9 approach, and observed the region of laser-induced CNV was significantly reduced. These findings demonstrated that genome editing that employs Cas9 RNPs to inactivate disease-causing WT genes has the potential for local targeted treatment of non-hereditary degenerative disorders [35].

CRISPR/Cas9 Technology Used in Cardiovascular Disease

Previously studies have shown that CRISPR/Cas9 was widely applied to treat cardiovascular diseases include coronary artery disorder, Wolff-Parkinson-White syndrome, hypertrophic cardiomyopathy, and calmodulinopathic Long-QT syndrome. LDL-C has been identified as a main cause of death from cardiovascular disease worldwide. Statin medications have been shown to control the risk of coronary artery disorder by suppressing LDL-C levels, but they come with a slew of adverse effects that typically lead to poor adherence. LDL receptors that are in good working order lower cholesterol levels. PCSK9 is an LDL receptor antagonist expressed in the liver, has become a popular genome-surgery target. Gain-of-function mutations in the gene have been linked to greater LDL-C levels, as well as an increased risk of hypercholesterolemia and coronary heart disease [36]. Previous studies reported that the loss-of-function mutation of the PCSK9 gene did not show adverse clinical consequences, but it reduced the LDL-C content and the risk of coronary artery disease [37]. This leads scientists to suspect that PCSK9 gene therapy could reduce the risk of cardiovascular disease.

Based on this, Ding et al. employed CRISPR/Cas9 system to knockout PCSK9 in mouse liver. They edited almost half of the PCSK9 alleles with no substantial off-target consequences. Furthermore, they found that the PCSK9 levels in the plasma of the edited mice was reduced by nearly 90%, while the total cholesterol levels in the plasma was decreased by 35–40% [38]. Similarly, Ran et al. used an AAV vector, which is more appropriate for human therapeutics, to target PCSK9 in mouse liver and found an effective knockout via NHEJ. This procedure reduced the PCSK9 content in the blood by 95%, while the blood cholesterol level also decreased by 40% [39]. Taken together, these and other studies indicated that employing somatic gene editing to lower blood/plasma cholesterol levels can help patients reduce their risk of cardiovascular disease.

Late-onset adult diseases can be caused by an autosomal dominant mutations in a single gene. Among them, MYBPC3 gene mutation leads to hypertrophic cardiomyopathy. Aberrant myocardial relaxation and ventricular hypertrophy are the characteristics of the disease, which ultimately leads to diastolic heart failure and arrhythmias

[40, 41]. Using the CRISPR-Cas9 technology, Ma et al. successfully repaired the MYBCP3 gene with a 4-bp deletion [42].

PRKAG2 cardiac syndrome (PS) is a rare autosomal dominant genetic disease caused by PRKAG2 mutations. Its main characteristics include myocardial glycogen storage, myocardial hypertrophy, ventricular pre-excitation, and patients have a higher risk of arrhythmia and sudden cardiac death. In patients with familial Wolfe-Parkinson-White syndrome, Xie et al. discovered that the histidine at position 530 of the PRKAG2 was mutated to arginine and constructed a mouse model with this mutation. The abnormal hypertrophy of the heart and increased glycogen storage in this model mouse indicates that the mutation is causally related to PRKAG2 syndrome. Subsequently, they used CRISPR/Cas9 system combined with AAV-9 to repair the mutated PRAKG2 allele and restore normal heart shape and function [43].

Calmodulin is a key Ca^{2+} sensor for cardiac function, its missense mutations caused calmodulinopathies. These patients are accompanied by life-threatening symptomatic arrhythmias associated with LQTS, including ventricular fibrillation and ventricular tachycardia. The mutation results in a significant overexpression of the protein, which causes the action potential to be prolonged. Therefore, Limpitikul' team employed CRISPR interference to reduce the expression levels of calmodulin, thereby shortening the duration of the action potential and lowering the effect of LQTS [44].

Application of CRISPR/Cas9 on Lung Cancer Therapies

Lung cancer is the most frequent cancer on the planet. Multiple genes and signaling pathways play an important role in the formation of lung cancer [45], and clinical treatment of lung cancer has been extensively studied. The term "cancer gene therapy" refers to a type of treatment that involves active gene alterations [46]. Lung cancer genome repair and suppressing the expression of certain proteins have become attractive ways for studying and treating the disease. In recent years, CRISPR/Cas9 technology has received widespread attention and has been used in the research of lung cancer.

Knockout overactivated, overexpressed, or mutant target oncogenes has the potential to be cancer therapeutic. Oncogenes such as CTNND2, FAK, RSF1, EGFR, and NESTIN have recently been investigated in the context of CRISPR/Cas9 gene editing for lung cancer treatment [47–53], [54]. These oncogenes can promote the occurrence and development of lung cancer, and enhance the ability of lung cancer cells to invade or metastasize.

Knockout mutant EGFR allele by using CRISPR/Cas9 system suppressed the growth and proliferation of lung cancer cells [47, 49], and reduced tumour sizes in xenograft mice implanted with lung cancer cells [47]. Using CRISPR/Cas9 system to knockout the FAK gene in KRAS mutant NSCLC cells leads to persistent DNA damage and radiation sensitization [50]. In lung cancer cells, knockout of NESTIN gene facilitate cell apoptosis, inhibit cell proliferation and invasion by suppress EMT

[54]. RSF1 deletion causes G1 cell-cycle arrest and promotes cell apoptosis, simultaneously inhibited cell proliferation and migration in lung cancer cells [51]. In lung adenocarcinoma, oncogene δ-catenin facilitates tumorigenesis. Using CRISPR/Cas9 to knockout the CTNND2 gene in lung cancer cells to deplete δ-catenin, leading to the inhibition of the Wnt signaling pathway, thereby eliminating the tumorigenic and metastatic ability of cancer cells in vivo [53]. In general, current study suggests that the CRISPR/Cas9 system for oncogene editing has the potential to become a lung cancer therapy.

Inactivation of tumor-suppressor genes, on the other hand, is also critical during carcinogenesis [55, 56]. Tumor-suppressor gene expression products can impede cell proliferation, enhance cell differentiation, suppress cell migration, and inhibit cancer development [57], [58]. The activation of oncogenes is caused by the mutation, loss of function, or knockout of tumor-suppressor genes, which leads to carcinogenesis. Many cancer types have certain tumor-suppressor genes that are altered and expressed at low levels, and these tumor-suppressor genes are key candidates for gene editing therapy using CRISPR/Cas9 system [26]. Tumor-suppressor genes can be repaired by employing the CRISPR/Cas9 technology, and their function and activity can be restored to prevent cancer. Targeted correction of inactivated tumor-suppressor genes using CRISPR/Cas9 system could also be useful in treating lung cancer. In a Kras-driven animal model of lung cancer, a CRISPR/Cas9 deletion of Keap1 resulted in overexpression of Nrf2 and promoted tumor growth and survival [59]. Xu et al. found that upregulating the mTORC2/Akt pathway facilitated cell survival, enhanced cell viability, and increased cell proliferation, metastasis and invasion in the lung cancer cells by genetic deletion of the tumor-suppressor gene mitofusin 2 (MFN2) [26]. The tumor suppressor miR-1304 can be genetically knocked out, which increases heme oxygenase-1 (HO-1) production and promotes cell survival and expansion [60]. Few researches have used the CRISPR/Cas9 gene-editing technique to investigate tumor-suppressor genes in lung cancer, which should be regarded an essential direction for future study. The use of CRISPR/Cas9 technology to repair and activate dysfunctional tumor suppressors could provide much-needed hope for cancer therapy.

CRISPR/Cas9 System Used in Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is a type of degenerative muscle disorder caused by genetic abnormalities that cause dystrophin in muscle fibers to be disrupted. This deadly illness has no cure. CRISPR/Cas9 technology has shown to be an effective tool for genetic modification and potential treatment. By using CRISPR/Cas9 system, Long et al. modified dystrophin gene (Dmd) mutation in DMD model mice. And they discovered that genome surgery lead to genetically modified mice with *Dmd*

gene corrections ranging from 2 to 100%. Furthermore, the degree of muscle phenotypic rescue outperformed the effectiveness of gene repair, indicating that corrected cells have an advantage and contribute to muscle regeneration [61].

By using CRISPR/Cas9 technology in DMD model, another work showed that a 23-kb genome fragment covering the mutant exon 23 was successfully deleted, restoring dystrophin production and the dystrophin-glycoprotein complex in mdx animals [62]. In dystrophic mdx^{4cv} mice, Bengtsson et al. performed the CRISPR/Cas9 gene-editing assay to totally repair the dystrophin mutation. They found that approximately 70% of the treated muscles expressed dystrophin and increased strength generation after intramuscular injection. Additionally, systemic delivery of the CRISPR/Cas9 system causes broad dystrophin expression in both cardiac and skeletal muscles [63].

On the other hand, recent research reports that the biogenesis processing region in pre-miR-29b can be effectively targeted by CRISPR/Cas9 plasmids though local injection of into the tibialis anterior muscle or gastrocnemius muscles. In mice, stimulation of the AKT-FOXO3A-mTOR signaling pathway reduced muscle atrophy, immobility, and denervation induced by angiotensin II (AngII), as well as AngII-induced myocyte apoptosis, resulting in considerably enhanced exercise capacity [64]. The CRISPR/Cas9 gene-editing technology has been widely employed to cure a variety of diseases and to learn more about human disease.

The CRISPR/Cas9 Technology in Organoid for Future Personalized Treatment

Humans suffer from around 18,000 different diseases, including single-gene and multi-gene diseases. Even if it is the same disease, the multiple mutations of multigenic diseases offer them a unique heterogeneity ability that may exist differences in different patients. However, this one-of-a-kind feature of variability posed a significant difficulty for therapeutic rehabilitation. The CRISPR/Cas9 system with simple, accurate and precise genome editing capabilities has aroused the interest of scientists all over the world. Mutations, deletions, malfunction, or overexpression of functioning genes are responsible for a wide range of disorders. Therefore, by repairing the sequence of the mutant gene to restore its normal function or reducing the expression of the target gene to restore it to a normal level in the future, individualized treatment of patients with CRISPR/Cas9 gene editing technology may be effective in curing a variety of diseases.

Conclusion

In summary, we described the potential of CRISPR/Cas9 editing organoid technology in disease model construction and disease treatment. The application of gene editing in organoids is expected to expand in the future. However, several problems remain linked to the performance of CRISPR/Cas9 technology, such as decreasing Cas9's off-target effects and methods of delivering sgRNA to target cells. As a result, it's critical to enhance Cas9 optimization and reduce off-target effects.

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Chapter 10
Ethical Implications on Organoid

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Abstract **Introduction**: Over the last decade, the number and exposure of studies creating and implementing organoid technology have skyrocketed. Despite its potential in science, organoid technology poses complex ethical challenges that may hinder future translational benefits for patients. To encourage ethically acceptable innovation for the benefit of patients, the interdisciplinary conversation between diverse stakeholders in organoid research and its translational advantages is necessary. Organoid technology poses numerous major ethical concerns, including cell source, informed permission of cell donors, the legal status of organoids, human "chimaera," gene editing, organoid transplantation, commercialisation, potential abuse, and long-term preservation in biobanking will be the central focus in this chapter. **Methods**: For the selection of literature, the MEDLINE/Pubmed database was used, particularly the MESH vocabulary. The keywords used in the MEDLINE research were: ethics in organoid; the ethical implication of organoid; ethics in biobanking; consent for organoid; gene editing in organoids; organoid transplant; legal status of the organoid. **Results**: Organoid technology has had a significant influence on biomedical research. The most significant impact comes from debates about ethical issues such as animal experimentation, the use of embryo cells, organoid transplantation, drug discovery, storage and biobanking, organoid accessibility, laws governing and curbing organoid misuse, and control over the genetic information of patients who donate cells for disease modelling purposes. However, organoid research poses additional ethical concerns that necessitate rethinking and maybe recalibrating ethical and legal laws. **Conclusions**: Progress in creating different organoids has revealed a slew of ethical concerns that necessitate moral and regulatory considerations. Moral arguments will revolve around the concerns of artificial life, animal humanisation, and the moral

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position of brain organoids and gastruloids. Regulatory issues highlight the need to agree on a set of rules governing clinical uses of organoids, biobanking, and consent.

Keywords Organoids · Ethical issues · Biobanking · Consent · Precision medicine

Abbreviations

3D	Three-dimensional
ECM	Extracellular matrix
ASCs	Adult stem cells
PSCs	Pluripotent stem cell
iPSCs	Induced-pluripotent stem cells
IRB	Internal Review Board
IEC	Internal Ethics Committee
CFTR	Cystic fibrosis transmembrane conductance regulator
HTS	High-throughput screening
CRISPR	Clustered regularly interspaced short palindromic repeats
ESCs	Embryonic stem cells

Introduction

An organoid is a three-dimensional (3D) multicellular in vitro tissue construct that closely resembles its corresponding in vivo organ, allowing researchers to examine features of that organ in a tissue culture dish. Today, the term organoid is most often used to describe constructions made from stem cells, which can be pluripotent or adult stem cells from diverse organs. The mechanisms that produce these tissues in vitro are similar to natural tissue formation or maintenance. The complexity of the organoid will be determined by the developmental capacity of the initial stem cells. Some approaches for making pluripotent stem cell-derived organoids leave the cells alone, allowing intrinsic self-organisation and presumably stochastic processes to design the tissue. Self-organisation occurs inside the organoid by spatially limited lineage commitment and cell sorting, necessitating activating different signalling pathways mediated by intrinsic cellular components or external environments like the extracellular matrix (ECM) and media.

Sato et al. demonstrated that adult intestinal stem cells expressing single leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) might generate 3D intestinal organoids without a mesenchymal niche Matrigel self-organise and develop into crypt-villus structures [1]. This was the first time a single adult stem cell (ASC) was used to make a 3D organoid culture. Adult epithelial stem cells expressing the

general Lgr5 marker may be grown under tissue-repair conditions to produce epithelial organoids directly from healthy and damaged organs such as the stomach, liver, lung, and pancreas [2]. Organoids produced from adult stem cells take advantage of the tissue regeneration process that these cells drive, and they may be grown directly from the epithelium of numerous organs, whether healthy or sick. Organoids can be tested using any method that has been established for cell lines.

History of Organoid

Organoids are cells that grow in vitro in a predetermined 3D environment to produce mini clusters of cells that self-organise and differentiate into functional cell types, mimicking the shape and function of an organ in vivo. Organoids cultures are derived from stem cells and can be crafted to replicate much of an organ's complexity or express selected aspects of it, like producing only certain types of cells [3–6]. Henry Van Peters Wilson demonstrated the first in vitro organism regeneration trial by showing that dissociated sponge cells could self-organise and regenerate into a whole organism [7]. In 1960, Paul Weiss and A.C Tayler experimented with reconstitution of complete organs from a single-cell suspension of chick embryos in advanced stages of differentiation. Their study demonstrated that the single-cell suspensions prepared from organs like kidney, liver or skin of 8–14 days chick embryos were able to give the remarkably complete and morphologically well-organised organs with the various tissue components. The results re-emphasise internal 'self-organisation' as one of the most basic problems in the study developments [8]. Move forward to 1981, and stem cell research began when the pluripotent stem cells were first isolated and established from mouse embryos [9, 10]. After the isolation and establishment of pluripotent stem cells, induced pluripotent stem cells (iPSCs) were established by reprogramming mouse and human fibroblast, which greatly impacted stem cells and organoid research [11, 12]. Cell culture conditions were improved by stimulating the in vivo microenvironment and demonstrated that the Engelbreth-Holm-Swarm tumour (EHS) ECM extract and the presence of ECM matrix were important cell-matrix interactions in tissue maintenance and differentiation [13, 14]. Shifting from 2 to 3D organoids cultures was done by generating cerebral cortex tissue from ESCs using the 3D aggregation culture method and showed that some proteins expressed in adult intestinal stem cells could form a 3D intestinal culture in an ECM matrix self-organised and differentiate [15]. Many organoid cultures were done on other systems, including stomach, liver, pancreas, lung, kidney, brain and retina, and they used either ASCs or pluripotent stem cells (PSCs). The population of stem cells in the small intestine was discovered, and the tissue host of stem cells was separated, allowing them to construct 3D gut organoids. The organoids culture contained all cell types found in vivo, and it can be maintained and grown in vitro with the help of growth hormones [1]. Using the same principles, more systems such as the stomach, liver, pancreas and others can be generated by ASCs derived organoids [15–19].

Application of Organoids

Organoids are one of the great tools in cell culture techniques in many biomedical studies. Various applications of organoids in many areas, including developmental biology, disease modelling, drug precision, regenerative medicine, drug discovery medicine, and toxicology [20–26]. The 3D organoid construct has also been employed to investigate the relationship between infectious pathogens and corresponding cancers [27]. Organoids derived from different mouse or human tumours that are being widely used to study cancer types. Previous studies were done by derived human liver cancer organoids from patients by extensive refinement of medium conditions to expand three common subtypes: hepatocellular carcinoma, cholangiocarcinoma, and a combination of hepatocellular cholangiocarcinoma [28]. Other than that, organoids have opened the possibilities for biobanking, and these represent a valuable resource for clinical application such as analysis for cancer stratification and drug screening for precision medicine [29, 30]. Tumour heterogeneity and clonal dynamic were preserved after the serial passage of organoids, indicating that these tiny or mini tumours are genetically stable with various clinical applications [31]. The potential organoids in precision medicine and regenerative medicine are promising, and it is important to mention and highlight the safety, ethical and legal concerns before moving to clinical application. Organoid technology's ethical difficulties have been examined, and specific ethical and regulatory control proposals have been made.

What Are the Ethical Issues?

Ethical issues on the development and usage of organoids are largely attributed to how such organoids attain functional and structural similarity to real human organs and thus represent existing ethical discourse for that real organ in a human. Such discussions revolve around the philosophical grounds, benefits towards animal exploitation, precision medicine, consent models, and moral status of brain organoids and gastruloids.

Philosophical Grounds

In our traditional philosophical construct, organs have always been part of organisms, both biologically and existentially. In organ transplantation, the idea of having somebody's organ transplanted into another has sometimes been perceived as the movement of existential personhood rather than a mere technical movement of a mechanic organ, which has delineated the moral argument against commercial organ transplantation [32].

Organoids presented an idea that an organ can be 3D-printed or developed into its functional 3D structure from cells outside of the human body, using artificial biomedical techniques, and then used for research and, potentially, clinical applications. This would present a deconstructive force towards the traditional idea of organ development through natural embryological processes. In such a way, the artificial creation of organoids through 3D printing may present the notion of *artificialisation of the living* [33]. On the other hand, the creation of organoids itself would present the notion of the *creation of life*, especially when it comes to organs associated with personhood and individuality, such as brain organoids and gastruloids. The notion of life inherent in the development of cerebroids and gastruloids will be presented as the moral status of these two special organoids is discussed. Further on this, the application of organoids in research whereby human organoids are transplanted into animals to study their biological characteristics or responses to drugs might spark the idea of humanising animals, especially when it involves cerebroids, such as in the case of Alzheimer's study [34].

Organoids have the potential to impact the ethical elements of biomedical research throughout the whole innovation cycle. These ethical concerns are comparable to those faced by regenerative medicine, which attempts to restore damaged function through the cell, tissue, organ repair, replacement, or regeneration [35, 36]. Animal experimentation is frequently used as a proxy for studying human tissue development, injury, repair, and the pathophysiology of human diseases, including drug testing. In this vein, the creation of animal models is a typical part of disease-based research techniques. By considering the 3R principles (replacement, reduction and refinement), which have achieved international recognition as a public policy in animal testing, a compromise may be struck between allowing animal experimentation and respecting animals [37]. Organoids are viewed as a replacement for the R-component of the 3R concept, which replaces animals with other techniques, however, they lack immune cells and do not represent immune system interactions. Organoid researchers must continue to stress that basic discoveries and therapeutic therapies cannot be produced without animals [35].

Organoids can imitate certain elements of the 3D design, cell-type composition, and functionality of genuine organs while retaining the benefits of simpler and conveniently accessible cell culture models. As a result, they have much potential in biological and medicinal applications. Regulating self-organisation generates organoids that develop deterministically, robustly, and physiologically relevant shapes and sizes, extending organoid lifespan to create mature, functional tissues that reach homeostasis; and replicating multi-factorial pathologies by incorporating additional key tissue compartments of native organs are all major challenges [38]. Overcoming these obstacles will need a multidisciplinary approach, with bioengineering lessons likely to be particularly useful.

Human organoids and gastruloids research raise ethical concerns about their origins and their current and future applications. Unique concerns such as the amount of maturation that may be accomplished in vitro or through chimaera research, as well as basic ethical questions like the provenance of human biomaterials and the use of gene-editing technologies, are among them [39]. Human gastruloids present

possible philosophical and ethical problems about the production of early human life due to their resemblance to embryos [39, 40]. Suppose human gastruloids are considered to be functionally equivalent to human embryos. In that case, a slew of ethical and regulatory questions arises, including whether it is appropriate to create these PSC-derived constructs in jurisdictions that prohibit the generation and destruction of research embryos, as well as the limits on how far human gastruloids can mature.

Although various forms of chimaera research, such as the transplantation of human cancer cells into mice or functional engraftment of cells derived from human PSCs, have been carried out without much controversy for decades, ethical concerns may arise when cells and complex in vitro structures of human origin are introduced into the brains or reproductive systems of animals. Concerns regarding unintentional cross-species fertilisation involving human and non-human gametes may arise due to the introduction of human gonad-like organoids into animal models. If integration into reproductive systems is possible, it will be critical to take steps to ensure that such chimeric creatures cannot reproduce [39, 41].

Human biomaterials must be obtained with explicit and voluntary informed permission commensurate with the planned use of the biomaterials and following local norms and laws. The federal research regulations in the United States currently allow research involving pathological or diagnostic specimens if these sources are publicly available or if the information is recorded so that subjects cannot be identified, either directly or through identifiers linked to the subjects' identities [42]. Tissue discarded during clinical procedures can be used for research without the patient's explicit consent, as long as the tissues are anonymised and the patient's admission form or consent for diagnostic or surgical procedures states that biomaterials collected during treatment can be used for "education and research" [42]. In the context of the Malaysian situation, researchers must obtain informed consent from patients, including biobanking, before collecting biological samples prospectively from patients undergoing regular examination or treatment. It is also worth noting that, while patients' agreement has been acquired for the use of the tissue in future research, any subsequent study must first receive clearance from the Internal Review Board (IRB) or Internal Ethics Committee (IEC) [43].

The Benefit Towards Animal Exploitation as Disease Modelling

Baertschi et al. [33] listed at least two ways how organoids may benefit in a way where pre-clinical studies could use fewer animals. First, organoids may provide an alternative (or even better) means and methods that could bypass the need for animal research, for example, toxicology study or efficacy testing of new drugs. Second, xenotransplantation may be abandoned because such an effort to breed genetically modified animals for the sole purpose of developing organs for human transplantations can be replaced by the development of clinically viable organoids.

However, the second benefit might still be hypothetical due to the essential lack of vascularisation and innervation in organoid development. Organoids have shown that cells can rearrange into sophisticated tissue-specific structures in the presence of modest inductive stimuli. However, the absence of hierarchical organisation and acceptable tissue size has been a key stumbling block in obtaining fully in vivo-like functioning [44]. A complex vasculature network interpenetrates and interacts with growing tissues in vivo, allowing for oxygen, nutrition, waste exchange and inductive biochemical exchange and a structural template for growth [44].

Organoid cultures for disease modelling benefit from simulating diseases at the organ level instead of conventional cell cultures of a single cell type. Furthermore, organoids generated from human ASCs or iPSCs might serve as models for human illnesses by recapitulating unique human characteristics important for translational research. The concept has opened up the possibility of drug testing and screening applications that organoids can simulate human diseases. Recently, drugs to treat Zika virus infections were tested in hPSC-derived cortical neural progenitor cells and validated in organoids and animal models [45]. This proves that organoids are a viable alternative for evaluating medicines against this disease. Organoids generated from a single patient with a highly uncommon cystic fibrosis transmembrane conductance regulator (CFTR) mutation for whom no therapy exists were used to evaluate current CF medicines. They responded to a medication previously used to treat other CF mutations, allowing the patient to get effective treatment [46].

Moral values should be valued and respected by only using them in studies conducted with morally viable techniques. Karpowicz et al. has outlined that the ethical viability of neural chimaeras should be permitted if the following rules are followed: [1] To achieve credible scientific results, researchers should be obliged to employ a minimal amount of stem cells from the human brain; [2] the host animal should not be too morphologically or functionally comparable to humans (to mitigate the risk of developing human-like neurological networks), and [3] to avoid the appearance of human traits in the specimen, such as dignity, only dissociated human stem cells should be utilised [47]. Boers et al. suggest that organoids be recognised as hybrids with unclear relationships to people, objects, bodies, technology, nature, and commodities. The technical transformation of human biological material into organoids gives rise to new intrinsic, relational, instrumental, and economic value. When organoids are swapped, this hybridity should be continually recognised [48]. Novel insights into the biochemical and genetic processes of complicated human neurological diseases such as microcephaly, autism, and Alzheimer's disease have already been discovered utilising patient-derived brain organoids. Novel insights into the molecular and genetic processes of complicated human neurological diseases, including microcephaly, autism, and Alzheimer's disease, have already been discovered utilising patient-derived brain organoids. The use of hiPSC technology in conjunction with small-molecule high-throughput screening (HTS) makes it easier to create new pharmacotherapeutic methods, while transcriptome sequencing allows for transcriptional profiling of patient-derived brain organoids. Utilising the advent of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 genome

editing, customised cell replacement treatment with genetically corrected hiPSCs has never been more promising [49].

Rapid advances in human organoids research, particularly in human cerebral organoids, have resulted in the development of so-called mini-brains in the lab, which have most of the characteristics and functions of a fully-formed human brain. Human brain organoids may acquire a primitive form of sentience, defined as the simplest type of awareness connected to fundamental pleasure and suffering experiences shared by many animal species [50].

The ability to assemble various organoids into multi-organoid complexes has previously been shown using organ-on-a-chip technology and microfluidics. For example, combining organoid and organ-on-a-chip technologies resulted in sophisticated multi-layer tissue models [51]. Even though multi-organoid complexes expand the possibilities for drug testing, drug discovery, and customised therapy, these humanised models bring new issues and need moral attention [52]. The ability of such human organoid complexes to absorb and respond to stimuli or display some form of autonomous activity may elicit strong feelings about their human-like moral standing, necessitating further safeguards against damage.

Source of Stem or Progenitor Cells

Organoids are made from foetal or adult tissues and embryonic stem cells (ESCs) and iPSCs. ESCs are pluripotent stem cells (PSCs) with a nearly limitless capacity for self-renewal and the ability to differentiate into any cell type in the human body. This feature enables ESC-derived organoids to be excellent in vitro developmental biology models. The inner cell mass of in vitro fertilised blastocysts is used to isolate ESCs. The use of ESCs in organoid technology raises significant ethical questions about the worth of human life and human dignity. Depending on the embryo's developmental stage, research on human embryos and subsequent use of ESCs in organoids can be morally permissible, but only under rigorous conditions of informed consent and adequate authorisation.

The creation of iPSCs provides a ground-breaking alternative to using ESCs. iPSCs are adult somatic cells that have been reprogrammed to have pluripotency similar to ESCs [11]. Adult stem cells may be reproduced in different organoids to resemble actual organs, according to subsequent investigations. While iPSCs may not be a perfect replacement for ESCs in organoid technology, they can surely help avoid the significant ethical and legal issues ESC usage brings. iPSCs can avoid the destruction of embryos while also addressing the serious problems of potential health hazards and recompense for egg donors. Although iPSCs are a valuable tool for stem cell treatment, they also raise ethical problems. It is possible that aberrant reprogramming occurs during the generation of human-induced pluripotent stem cells, and the stem cells become malignancies during stem cell treatment [53]. The unlimited differentiation potential of iPSCs, which can be used in human reproductive cloning, is a major ethical issue, as it poses a risk of generating genetically engineered

human embryos and human-animal chimaeras, while unwanted differentiation and malignant transformation are major safety concerns [54].

Consent Models

The application and development of organoids involve procuring and storing human biological materials. As in biobanking, future use of these materials is often unavoidable. Individual consent before material sampling is a requirement, be it for research or clinical diagnostic purpose. A tiny amount of biological material might have considerable research value as it can be used for multiple studies and sustain long-term storage. In this regard, a one-off study-specific consent would not serve such a scenario, nor a blanket consent that provides limitless freedom to future use. There have been debates on various consent models, namely broad consent and dynamic consent.

In broad consent, sample donors provide a one-off consent to an array of broad research purposes and a governance model on the regulatory pathways of how the samples will be managed [55]. Although this consent model is currently deemed the most practical and widely used in biobanking practices, it has weaknesses. Such broad consent presented paternalistic governance on the future usage of the sample and provided little to no room for donor's autonomy and participation in decision making. An alternative method that employs continuous and real-time participation of donors was presented, a dynamic consent model. Dynamic consent is an online platform to facilitate personalised consent and two-way communication between researchers and research participants (sample donors). In dynamic consent, an initial broad consent might be obtained at the time of sampling, and donors may provide fresh consent to new research activities that were not foreseen before [56]. Given the nature of the model, such a platform would limit participation into those that can accommodate the online platform, and this will be difficult in low and middle-income countries setting. Such a model has also been deemed impractical and burdening researchers as well as research participants [57]. Indeed, donor surveys indicated that only a few demanded recurrent, project-specific consent and wished to place limits on the uses to which their tissue could be put [58].

Genetics Modifications and Precision Medicine

Although this is still largely hypothetical, progress in developing organoids from autologous cells provided an insight that clinical translation is a possible future. The development of Patient-derived tumour organoids (PDOs) [59] and intestinal organoids to model cystic fibrosis [60] opened up pathways for the application of precision medicine through organoids. Such applications would allow testing of candidate drugs to ensure benefit before being administered to a particular patient.

When using this approach, it is critical to maintain linkages between the patient and the organoids, which obviously can compromise measures to protect privacy. In addition, organoids will provide a new form of evidence to support the effectiveness of drugs for individual patients, challenging existing models of obtaining clinical evidence and reimbursement [61].

Human organoids can be used with the genome (or gene) editing technologies to investigate diseases and create new treatments. Gene editing methods may be used to change the expression of genes in ESCs, iPSCs, germ cells, somatic cells, and even human embryos, and they have much therapeutic promise. Furthermore, genome editing technologies can help represent uncommon genotypes in organoid development. Donors with unusual or uncommon genotypes may be highly important in organoid technology, but this puts them under ethical pressure to contribute to their cells. Off-target effects can cause unintended mutations at many loci, raising worries about the safety of this genome editing method, which has the potential to cause cancer. This is especially true when organoids or cells generated from organoids are intended for in vivo therapeutic applications, when genomic integrity is jeopardised, posing severe ethical issues [52].

Organoids have swiftly gained popularity as a model for bridging the gap between in vivo animal models, which are time-consuming and expensive to maintain, and in vitro two-dimensional cell culture methods, which lack 3D tissue structure and frequently include cancer-related genetic changes. They may also be utilised for disease modelling and treatment development, for example, by forming organoids from cancer and diseased tissues [62–67]. Organoids have been subjected to various genetic engineering techniques, resulting in a new field known as organoid genetics. These techniques allow for precise changes to the genomic DNA sequence. If the changes are made to a coding sequence, they can cause a specific change in the target protein, revealing information about the biological function of a particular residue or the protein as a whole. Two key factors must be considered in this process: the genetic tools and how to deliver them to the target cells. It would be useful to check the factors to consider when selecting the delivery method and genetic engineering tools. The sort of organoid system to be utilised and the type and purpose of editing should all be considered [68]. Adult stem-cell-based organoid cultures are emerging for growing primary normal and diseased tissue in vitro for lengthy periods. This 3D organoid culture, when combined with genome editing techniques, has much potential for studying human liver and pancreatic biology, as well as the molecular processes behind disease onset and development [69]. The ability to manipulate adult stem cells in vitro through genetic alteration of these organoids might help researchers better understand human biology and provide gene repair for regenerative medicine.

Arteginani B and colleagues have reported that CRISPR-HOT stands for CRISPR-Cas9-mediated homology-independent organoid transgenesis, allowing for the rapid creation of knock-in human organoids representing various tissues. CRISPR-HOT eliminates laborious cloning and surpasses homology-directed repair (HDR), which was previously utilised to boost HDR-mediated knock-in in obtaining accurate integration of foreign DNA sequences into targeted loci [70]. Organoids made from ASCs can be utilised to simulate genetic illnesses like cystic fibrosis and cancer.

Using CRISPR/Cas9, a bacterial defensive mechanism, Genome editing has recently emerged as a simple and reliable laboratory technique. Organoids and CRISPR/Cas9 work together to open new ways to research organ development and human illness in vitro [71].

Organoid Biobanking

The development of organoid biobanks for various diseases is an emerging use for organoids. Such biobanks will eventually aid in developing sophisticated screening platforms that cover a wide spectrum of genetic variation in the global population. The creation of biobanks maybe even more critical in cancer, a disease characterised by an almost infinite number of mutations. A biobank of primary colon cancer organoid lines from 20 individuals was created in one research, with the original tumours' histological and major genetic characteristics maintained [63]. In addition, a proof-of-concept drug screening revealed that the pharmacological responses of organoids with various mutations matched previous clinical findings. A biobank of 55 distinct colorectal tumoroids covering various histological subtypes and clinical stages was created [72]. The number of tumour biobanks is growing rapidly: A biobank of gastrointestinal cancer organoids was recently utilised to evaluate *patients*' in vitro *and clinical treatment responses.*

Organoids are an intriguing and cutting-edge tool for drug research and precision medicine, and biotech and pharmaceutical firms are highly interested in them. Off-the-shelf organoids will be required if organoid transplantation progresses to the clinical stage. Organoids are complex entities connected with many types of biological material (such as tissue samples, cell lines, and whole organs), and they have a genetic and functional relationship to the donor. The moral position of organoids and how organoids are connected to or allude to donors is essential since they can impact the ethical assessment of the amount of commercialisation of organoid biobanking. Patients' agreement is necessary for collecting biological materials, and others argue that using these tissues for research is unethical. As a result, consent is typically unnecessary if the tissue is deidentified because the risks are unlikely to be realised. However, whether deidentification justifies and guarantees research on human tissue is a point of contention. These consent issues are addressed by organoid biobanking, which is a confluence of these technological advancements. Organoid biobanking is a promising and exciting new subject with much promise in scientific research, precision medicine, and regenerative medicine. It is critical to include all stakeholders in the discussion about developing adaptable governance systems. This includes donors' active and significant engagement [73].

Brain organoids and Gastruloids presented unique challenges in terms of their moral status. The moral status of brain organoids largely depends on their functional similarities with the real human brain, while the moral status of gastruloids depends on their extent of maturation to the level where all three embryological layers, namely the ectoderm, mesoderm and endoderm, are ready for differentiation. Although the

near-human complexity of complete brain organoids is still hypothetical, current progress with the development of various brain regions showed that this is a possible future [25].

Conclusion and Future Direction

Progress in the development of various organoids has pointed towards a plethora of ethical issues that warrant debates on moral and regulatory grounds. Moral debates will centre around the issues of artificialisation of the living, humanising animal, and moral status of brain organoids and gastruloids. Regulatory concerns delineate the need to agree on a set of regulations on clinical applications of organoids, biobanking, and consent.

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