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A REVIEW OF POTENTIAL LUNG ORGANOIDS EXPLORATION AND CLINICAL APPLICATIONS

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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Review Article

ABSTRACT

In recent years, the mortality rate remains unacceptably high despite many advancements made in the field of lung-related diseases. This can be attributed to the lack of current preclinical models and the inability to translate fundamental epithelial studies into clinical therapy. Three-dimensional cultures have properties present in stem cells, such as the ability to self-organize in matrices and able to generate structures that can be reprogrammed to represent an organ or a pathology. By adding source tissue ranging from cells to tissue fragments to a support matrix and specialized media, the generation of 3D organoid cultures are achieved resembling the physiological environment of the tissue's origin. Depending on the source tissue, growth factors, and inhibitors provided, organoids can be programmed to recapitulate the biology of a system and the progression of a pathology. In this review, the main objective is to discuss recent technical advances that efficiently use organoids as a tool for disease modeling, regenerative medicine, toxicology studies, therapeutics and various techniques used for large scale organoid generation. Using animal models for drug screening and toxicology studies has certain drawbacks, but organoids can help to overcome these obstacles. The advantages of stem cell-derived organoid models in comparison to current culture systems are highlighted, recent developments in tissue-based organoids are becoming specialized models for studies related to human translation medicine.

Keywords: Lung progenitor cells; magnetic-activated cell sorting (MACS); fluorescence-activated cell sorting (FACS); regenerative medicine; Induced Pluripotent stem cells (iPSC) and % Colony Forming Efficiency (%CFE).

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1. INTRODUCTION

The American Lung Association reports lung cancer as one of the leading causes of cancer-related deaths in the United States [1]. At some point in their lives, about 541,000 Americans living today will sometimes be diagnosed with lung cancer [2]. Large-scale genomic studies indicate that patients have intertumoral and intratumoral heterogeneity that demonstrate both genomic and phenotypic diversity [3]. Although patient-derived tumor xenografts (PDXs) have emerged as a useful model for translational research, limitations such as tumor heterogeneity in the PDX model and stromal cells that were initially present in the dissected tumor will be gradually replaced by host stromal cells have an impact on the study of human tumor-stromal interactions. This can be resolved through three-dimensional organoids, which closely recapitulate the morphological and genetic/epigenetic characteristics of the original tumor [4]. When cultured in two-dimension, cancer cell lines do not preserve the initial variability and complexity. However, three-dimensional organ structures are able to mimic the lung cancer more realistically. Due to their more authentic nature, epithelial organoids have been used for many applications, such as studying fundamental cell biology, drug screening across a range of lung diseases, toxicology tests on cells, and regenerative medicine.

Recent studies have identified biological processes specific to the human body and cannot be modelled in other animals such as immunodeficient mice, as their models do not properly reflect human physiology. These include brain development, metabolism, and the testing of drug efficacy. The emergence of human in-vitro 3D cell culture approaches using stem cells from different organs has gained widespread attention, as they have the potential to overcome these limitations. Human organoids can be seen as a novel experimental model that proves more reliable than the animal models being currently used [5-8]. Understanding the growth needs of the various stem cell populations, which can be accomplished by studying cell-cell interactions in organoids, can help to address the failure to meet the requirement of lung transplantation. There are many distinct cell types in the lung , which makes it a complex organ. A key research question is how these cells interact with development, homeostasis, and disease, and organoids provide a platform to investigate certain cell-cell interactions [9]. Therefore, the production of human in-vitro 3D cell cultures using stem cells from various organs is widely regarded as capable of overcoming these constraints.

2. ORGANOIDS AND CULTURING METHODS

Organoids are three-dimensional structures grown in cell culture that resemble the organ from which it originated. In-vivo, epithelial cells live in a complex organ microenvironment, supported by stromal cells and the adjacent extracellular matrix. Epithelial cells cultivated in a typical two-dimensional manner have only some physical and molecular similarities to the organ from which it developed. It also lacks the cellular heterogeneity found in the tissue of its origin. Organoid cultures typically rely on cell-cell interactions, between a stem cell and a putative stem cell niche, for their growth and differentiation. Epithelial progenitor cells isolated from the human lung and cultured in trans well cell culture inserts with stromal support, recapitulate in-vivo cellular interactions and represent the architecture of the tissue of its origin [10,11]. The earliest mention of a three-dimensional epithelial organoid model was described by Dr. J D Hackney in an Aspen Emphysema conference more than 40 years ago, but their utility in translational medicine remained limited until recently. The early days of organoid model usage required large amounts of starting materials and limited in-vitro viability were the regulating factors for high-throughput screening [12,13]. But due to the improvements made throughout the years, organoids from various endoderm-derived organs from humans, including colon [5], prostate [14], intestines [15], have been successfully cultured and used in various screening studies.

2.1 Culturing Organoids from Lung Tissue

Described the methods for isolating the distal and proximal lung epithelial stem cells and culturing organoids in a recent published article from our lab [16]. In the current study, we used the Magnetic-Activated Cell Sorting (MACS) method for depleting the blood cells(CD235a), immune cells (CD45), and endothelial cells (CD31), as well as, used the Fluorescent Activated Cell Sorting (FACS) for isolating epithelial cells, in particular HT II 280^{+} cells as distal lung stem cells and $NGFR^{+}$ cells as proximal lung stem cells. Distal lung cells express HT II 280 and SPC surface markers and Proximal lung cells express K5 and Sox2 surface Markers (Fig. 1). We described the methods of culturing alveolar and airway organoids using the isolated distal and proximal lung stem cells, respectively. Organoids derived from lung progenitor cells are considered to be clonally derived structures. In our previously published study, we showed this using genetic lineage tracing approaches, in which GFP negative, GFP low and GFP high lung epithelial progenitor cells self-renew to form clonally distinct organoids [17,18].

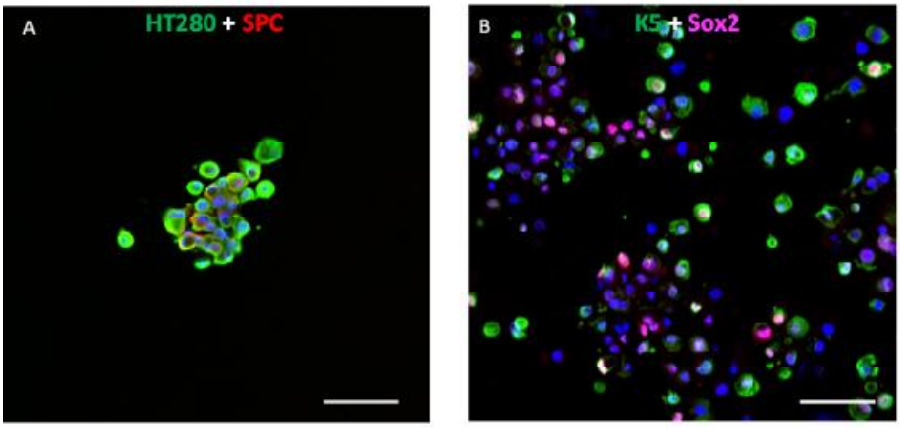

Figure 1: Characterization of distal and proximal lung organoids. (A) Immunofluorescent staining of corresponding distal organoids cultured in ALI medium showing HTII-280^{+} AT2 cells (green) and SPC (Red). **(B)** Immunofluorescent staining of corresponding Proximal organoids cultured in ALI medium showing K5 positive cells (green) + Sox2 (Pink). The marker used for isolation of AT2 cells in this study, HTII-280 and NGFR for proximal cells. Scale bar = 50um.

Epithelial-mesenchymal interaction is critical for the successful formation and differentiation of organoids from human lung progenitor cells. The MRC5 cell line is convenient to use, capable of 42-45 population doublings before the onset of senescence and grows cells rapidly enough for the generation of high-quality organoid cultures. Co-cultured epithelial cells with primary fibroblasts isolated from adult lung tissue, as well as embryonic lung tissue in the lab. Observed that the results are consistent with the use of MRC5. It is possible to use another normal lung fibroblast cell line, such as CCD-34-Lu. However, further testing must be done to determine their efficiency regarding organoid growth.

In our experience, the seeding density is critical for the formation of quality organoids with higher colony forming efficiency % (CFE%), as well as for the organoid size (100-500 µm). Seeding higher numbers of cells limits the space available for the organoids to expand, and the cells form small clusters, fail to polarize, form a lumen, and differentiate into specialized cell types that can secrete surfactant or mucus into the lumen.

Repeated the experiment multiple times with different biological samples. Started organoid culture with 1000 cells/well, 2000 cells/well, and 5000 cells/well. The colony forming efficiency, shown in Fig. 2, is from two different biological samples with three technical replicates (5000 cells/ well). The variability in Percentage of Colony Forming Efficiency (%CFE) can be up to 2-5% and is dependent on parameters, such as donor age and donor health. %CFE can also be affected by the amount of time required to procure the tissue. Cells from fresher tissue samples exhibit higher %CFEs. Organoids were cultured in different media, such as Pneumacult ALI (Air- liquid interface) medium and Small airway epithelial cell growth medium (SAECGM) from Promo cells. Both medias have resulted in a similar %CFE (4-5%) but has differences in the size of the organoids. Organoids cultured in ALI medium are larger in size and have greater lumen, they are around 500µ, whereas SAECGM is around 100 µ.

To further enhance the colony forming efficiency, we tried to culture organoids by adding either individually or in combination of 10 µM Rho kinase inhibitor (ROCK) and 10 µM TGFβ inhibitor (Immunosuppressive cytokine). Based on our results, %CFE is higher when organoids were cultured with 10 µM Rho kinase inhibitor for the first 24 hours after seeding and with 10 µM TGFβ inhibitor from day 2 to day 15 (Fig. 3). Organoids were passage between day 25 to day 30. The %CFE almost doubled in Passage 1 (P1) compared to Passage 0 (P0).

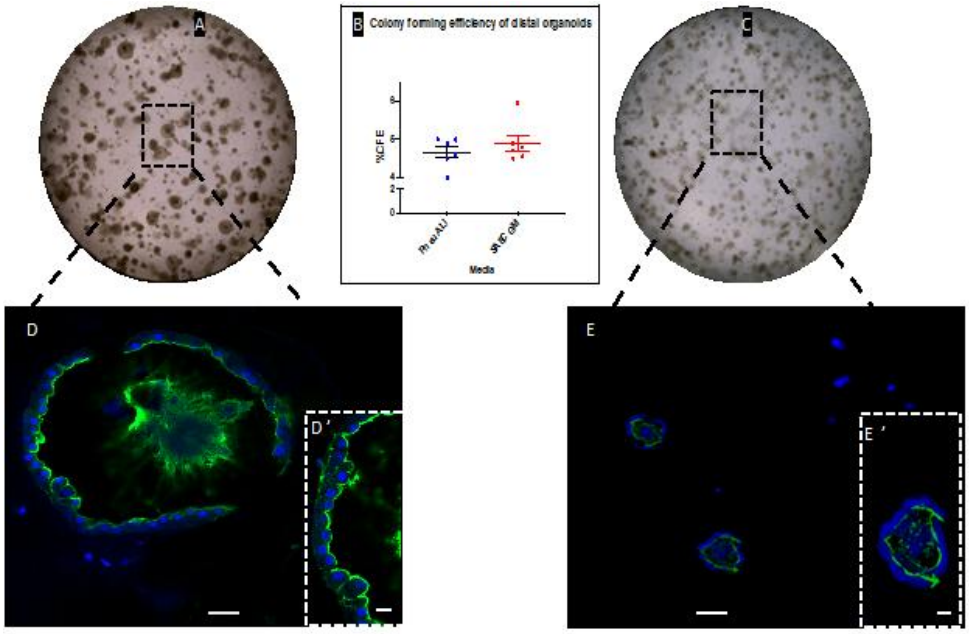

Figure 2: Colony forming efficiency and characterization of distal lung organoids (5000 cells/well). (A) Representative image of the human distal organoids cultured in PneumaCult-ALI medium (2x magnification). (B) The Colony forming efficiency (%CFE) was calculated on triplicate wells of organoids derived from two different biological samples. (C) Representative image of the human distal organoids cultured in SAGM medium (2x magnification).(D, D') Immunofluorescent staining of corresponding distal organoids cultured in ALI medium showing HTII-280+ AT2 cells (green). (E, E') Immunofluorescent staining of corresponding distal organoids cultured in ALI medium showing HTII-280+ AT2 cells (green). The marker used for isolation of AT2 cells in this study, HTII-280 Scale bar = 50um.

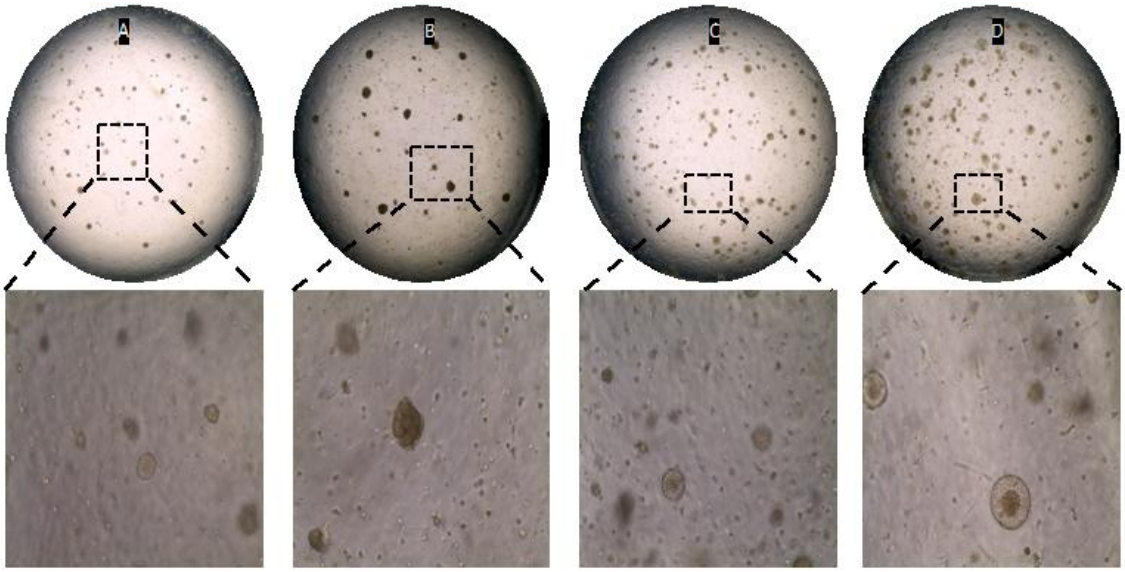

Figure 3: Culturing distal lung organoids (day10 1000cells/ well). (A) Representative image of the human distal organoids cultured in PneumaCult-ALI medium (control group). (B) Representative image of the human distal organoids cultured in PneumaCult-ALI medium + 10μM TGFβ inhibitor. (C) Representative image of the human distal organoids cultured in PneumaCult-ALI medium + 10μM Rock inhibitor. (D) Representative image of the human distal organoids cultured in PneumaCult-ALI medium + 10μM TGFβ inhibitor + 10μM TGFβ inhibitor. 2x Magnification

2.2 Generating Organoids from Expanding Lung Epithelial Tips *In-vitro*

In order to isolate the organoids, placed the tip of the distal epithelium in Matrigel with culture conditions similar to embryonic mouse lung. In the Matrigel, immune cells and red blood cells are inhibited, allowing the enhancement of selective population, such as progenitor cells. Human and mouse lung tips are analogous [19]. Resected, healthy or tumor material or biopsies can be used to generate organoids. Patient-derived organoids generated from biopsies provide a powerful resource for a variety of medical and translational approaches. In order to realize the potential of personalized medicine, it is useful for the study of drug toxicity and effectiveness [20].

2.3 Culturing Organoids from iPSC

The organoid culture, derived from human induced pluripotential stem cells (hiPSC), was stemmed from the manipulation of various multilineage differentiation and morphogenesis. Organoids derived from hiPSC are superior to 2D cultures in architecture, functionality, and has similar features of tissues as in vivo (microenvironment). We are showing the applications of these hiPSC derived 3D organoids in modeling cancer, hereditary diseases, host-microbe interaction. Organoids also help in discovering diagnostic markers for early disease detection through screening.

The availability of cadaveric tissue, a small source of donor tissue, is highly ineffective and logistically difficult for both transplantation and disease modeling; this often contributes to the low efficiency of the available cells/tissues. The main focus is on the pluripotency in human Embryonic Stem Cells (hESC) in the presence of specific culture conditions, as significant amounts of somatic transplantable cell types in vitro need to be obtained. Since hESC is self-renewable and proliferative, it can be manipulated to model human diseases. These cells can be used for transplantation as well [21].

Rossant and colleagues published an initial summary of lung organoid generation from iPS cells (Induced pluripotent stem cells) and included an air-liquid interphase in the final stage. In the processes of developing foregut endoderm, two populations of NKx2-1+ progenitors play an important role in developing the postnatal lung. TGFß and BMP inhibitors, combined with the stimulation of BMP (Bone morphogenetic protein)and the signaling of FGF (Fibroblast growth factor), helps generate the endodermal progenitor cells that are used for culturing organoids [22]. The Wnt signaling pathway plays an important role in regulating the Proximodistal epithelial patterning. Wnt activation withdrawal results in the promotion of proximal epithelial cells from primordial NKx2-1+ progenitors, as opposed to distal epithelial cells. Inversely, in the presence of Wnt activation, distal epithelial cells are enhanced [23]. Alyssa J. Miller and eta., addressed the schematics of the protocol and it's timeline for generating organoids from iPSC; their applications and limitations were discussed as well [24]. The organoids derived from very well-established differentiation protocols have the capacity to culture in-vitro, recapitulate developmental programs, form sophisticated 3D structures, and self-organize [25-27].

3. CLINICAL APPLICATIONS OF ORGANOIDS

3.1 Organoids as a Tool for Studying Fundamental Cell Biology

3D organoids help in research accurately a variety of in vivo biological processes such as tissue renewal, the function of stem cell or niche, the tissue response to drugs and mutations [28]. Attempts were made to model human organs biology – in 2D from human stem cells, either with or without a 3D matrix, human cell bio-printing and cell culture in microfluidic device("organ-on-a-chip") [29] before the advent of organoids and showed a potential for drug screening or research in human diseases [30]. 3D culture structures which are very similar to real human organs from which its originated and in some cases histologically undifferentiable from them. The common feature of any organoids, which is that it is produced through the in vitro imitation of human development or organ regeneration from pluripotent stem cells (PSCs) or adult stem cells (ADSCS) [5,6,31-35].

The study of organ formation can thus provide useful insight into processes underlying human development and regeneration of organs which focus on their importance as well as their possible use for fundamental biological research in the medicinal and molecular testing of drugs. This information might lead basic biologists to explore the use of this new platform to study human pathophysiology. Organoids has advantages in studying fundamental biology such as recapitulation of developmental biology, genetic manipulation, genome-wide screening. Human lung organoids are ideal for studying mesenchymal and epithelial interactions due to the presence of mesenchyme , epithelial cells and airway structures which helps in understanding human lung development, developing models for malformations in

human fetal lungs using patient-specific cell lines(24). Clonal organoids retaining airways and/or alveolar lineaging cells are generated from uninjured club cells in 3D-ALI organoid culture of club cells or club cell subsets, including BASCs (Bronchioalveolar stem cells). In vitro organoid culture conditions therefore promote the activation of the program of stem or progenitor cells, providing a strong model for investigating the initial reprogramming phases of differentiated mature cells into an undifferentiated condition [18,20,36].

3.2 Organoids in Regenerative Medicine

Crosstalk between endothelial cells and epithelial or stromal cells during development leads to a fully patterned organs such as lungs, kidneys etc. understanding cell-cell crosstalk during development can be applied therapeutically through the generation of transplantable miniature organ-like tissue called "organoids" [37]. The same objective is explored by regenerative medicine and organ transplantation to substitute diseased organs with new ones. While significant progress in regenerative medicine has been made so far, there is still insufficient current knowledge and organ engineering in its implementation, and organ regeneration remain inaccessible objectives [38-40].

Organoids offer an alternative to the supply of autologous tissue for whole organ and cell transplantation. Organ transplanting procedures such as renal transplant, lung transplant, and liver transplant, with high demands and poor success levels, may be improved by using organoids from that corresponding healthy organ from the same patient. Kidney organoids were successfully transplanted to adult mice by Taguchi et al., paving the successful way for the potential for organ transplants [41,42]. Transplanted cells benefit the patients with two distinct ways such as cell-engraftment and bystander effect. The first one involves, incorporation of cells in the damaged tissue epithelium of cells, such as stem cells/ progenitor, where expansion and differentiation will substitute for damaged epithelium. Transplanted cells help in tissue integrity improvement and disease outcome. In comparison, the second one involves in modulation of the host tissue through cues such as paracrine signaling helps in the promotion of endothelial and epithelial repairs [43]. The transplant of mesenchymal stromal cells (MSCs) has been investigated to assist repair of pulmonary diseases, including ARDS (Adult respiratory distress syndrome), COPD (Chronic obstructive pulmonary disease) and BPD (Bronchopulmonary dysplasia) [44-46]. Disease modeling for degeneration and developmental disorder can be achieved using organoids by introducing patient mutations in pluripotent stem cells using genome-editing techniques [47,48].

3.3 Lung Cancer Organoids Derived from Patient Use as an In vitro Cancer Model for Drug Screening

Lung cancer is histologically diverse and comprises three major types (Adenocarcinoma, squamous cell carcinoma, and small cell carcinoma) and several fewer common types (including adenosquamous carcinoma and large cell neuroendocrine carcinoma). The investigation of molecular specific treatment based on genetic changes was essential for classic in-vitro cancer cell line models because they offer significant manipulation advantages, time, and performance [47].

In general, cancer cell lines usually don't retain original heterogeneity and 3D structure; hence the nature of the lung cancer is fundamentally limited. PDXs recapitulate the tissue structure of the original cancer and preserve the original cancer genetic and histological features and has its limitations such as poor success rate, cost incur in producing and most importantly its resource-intensive and requires long term [3,49,50].

The creation of personalized lung cancer medicine will help in vitro lung cancer models representing individual patients. Effective lung cancer organoid generation that typically recapitulates the original patient tumor properties and preserves the histological characteristics of the original cancer tissue. New findings indicate, the lung cancer organoids are beneficial tool for drug discovery and new clinical trials and also used for drug toxicity predictions in non-cancer cells. Considering the short length of time to establish organoid cultures and screening, this model is used wider for preclinical studies in predicting drug responses in patients [51].

3.4 Usage of Organoids in Translational Applications and Personalized Treatment

Use of organoids models to a deeper understanding of cell activity during regeneration and disease is intended to increase the knowledge to the development of improved translational therapy [52]. In human lung organoids, CRISPR/Cas9 technology has been used to model and correct many lung diseases successfully [53-55]. Successful studies achieved by gene correction of SFTPB gene (Surfactant protein gene), HPS2 gene mutation (Hermansky pudlak syndrome type 2) using CRISPR/Cas9 in alveolar and human pluripotent stem

cell organoids [54,56]. Organoids will more precisely predict and act as a successful drug-screening tool including efficacy analyses, toxicity test, and pharmacokinetics analysis. Moreover, the potential tailored care methods for each individual patient can be archived by studying organoids [57]. Recent studies have shown that tissue restoration conditions can develop epithelial organoids directly from healthy and diseased bodies such as the lung, which carry the generic LGR5+ markers (Leucine-rich repeat-containing G-protein coupled receptor 5). Organoid technology opens up a range of methods for studying growth, physiology and disease, drug discovery, and personalized medicine. In the long run, cultured organoids will replace donor transplants and delivers gene therapy promises.

Organoids can also be isolated by using minimally invasive techniques such as fine-needle aspiration (FNA) from patients with very minimal processing without damaging the histologic growth patterns and infiltrating immune cells [58].

These Organoids are used as powerful tool for studying drug response studies. Multiplying the organoids by passaging them repeatedly up to 4-6 times and study the lung biology by performing 10x Single cell RNA-seq experiments which helps in developing new drugs for disorders such as Cystic fibrosis, Idiopathic pulmonary fibrosis etc.

4. CONCLUSION

In these studies, we analyzed several approaches using 3D organoids cultured from lung epithelial progenitor cells. For both basic and translational research, including human disease models and drug screening, we also outlined some of the potential uses of organoids in the fields of regenerative medicine and to study fundamental biology, such as tissue renewal, genetic manipulations and resolve unanswered issues in the biology of the lungs. Organoids have great potential for translational research, including personalized treatments, toxicology studies, and drug screenings. Organoids can be used to study developmental disorders and degenerative diseases such as cystic fibrosis, hypertrophic emphysema, which can also lead to potential reductions in usage of animals in research. Individuals will hopefully have opportunities for personalized treatment regimens by using patient-derived organoids derived from biopsies that can provide an exceptional source of tissue at both the site of disease and normal adjacent tissue. Phenotypic profiling and genetic alterations can be revealed, which in turn help in generating personalized therapeutic approached. Organoids generated from normal tissue adjacent to the site of disease can be used to reduce toxicity and side effects of a proposed therapy.

Despite the demonstrated benefit of organoid usage in the recapitulation of cellular heterogeneity and complexity, the usefulness of organoids in in-vivo research on dynamic interactions is still unknown. They lack surrounding stromal cells in the culture, failing to recreate the tumor microenvironment, which comprises fibroblasts, endothelial cells, immune cells, and extracellular matrix. In this case, the field will most likely overcome this limitation by synergistic engineering of organoid and lung-on-a-chip approaches, resulting in organoid-on-a-chip. One of the most difficult challenges is achieving complete organoid differentiation and culturing without Matrigel or growth factors. The presence of Matrigel can impact functional/biochemical assessments and in comparison, with 2D cell line culture, complicate cell harvesting and passing. In addition, enriched organoid growth factors can affect the natural tissue morphogen gradients, which can be rectified by trying culturing lung organoids in bioreactors without Matrigel. In order to successfully explore the potential of lung organoids in clinical medicine, much research needs to be done to overcome the above-mentioned hurdles.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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