*Review*

# Advanced lung organoids for respiratory system and pulmonary disease modeling

Journal of Tissue Engineering
Volume 15: 1–24

DOI: 10.1177/20417314241232502
journals.sagepub.com/home/tej
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**Abstract**

Amidst the recent coronavirus disease 2019 (COVID-19) pandemic, respiratory system research has made remarkable progress, particularly focusing on infectious diseases. Lung organoid, a miniaturized structure recapitulating lung tissue, has gained global attention because of its advantages over other conventional models such as two-dimensional (2D) cell models and animal models. Nevertheless, lung organoids still face limitations concerning heterogeneity, complexity, and maturity compared to the native lung tissue. To address these limitations, researchers have employed co-culture methods with various cell types including endothelial cells, mesenchymal cells, and immune cells, and incorporated bioengineering platforms such as air-liquid interfaces, microfluidic chips, and functional hydrogels. These advancements have facilitated applications of lung organoids to studies of pulmonary diseases, providing insights into disease mechanisms and potential treatments. This review introduces recent progress in the production methods of lung organoids, strategies for improving maturity, functionality, and complexity of organoids, and their application in disease modeling, including respiratory infection and pulmonary fibrosis.

**Keywords**

Lung organoid, bioengineeing platform, cellular niches, respiratory infection, pulmonary fibrosis

Date received: 8 November 2023; accepted: 30 January 2024

## Introduction

The lung, a vital organ responsible for the fundamental function of breathing, directly influences human life and survival. Impairment caused by pulmonary diseases significantly impacts life expectancy and overall quality of life. In particular, the recent global pandemic caused by coronavirus disease 2019 (COVID-19) has heightened public awareness of respiratory diseases, sparking a surge in interest in studying human lung mimetics to better understand and combat these pathological events. Lung organoid, a miniaturized three-dimensional (3D) cellular structure that recapitulates lung tissue, has garnered increasing attention worldwide owing to their unique features and advantages. Lung organoids offer a higher level of tissue complexity and heterogeneity compared to traditional two-dimensional (2D) cell models, and they provide a faster and more ethical alternative to animal models while closely simulating human pulmonary physiology.[1–3] Furthermore, organoids have been easily modified with gene editing systems like clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9), enabling the repair of mutations and modeling genetic diseases.[4–7] Despite these advantages, lung organoids still face several limitations, prompting ongoing efforts to overcome these challenges.

One limitation of lung organoids is their inability to fully replicate the cellular composition of actual lung tissue. For instance, in the lung tissue, there are various cell types such as endothelial cells, mesenchymal cells, immune cells, and specialized epithelial cells which are specific to different regions. Addressing this limitation

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requires a comprehensive understanding of the diverse cell types present in actual lungs, their differentiation mechanisms, and optimizing culture conditions. Recently, the technological advancements in genomics, including single-cell RNA sequencing (scRNA-seq) and spatial transcriptomics, have provided unprecedented insights into the intricate cellular heterogeneity of the respiratory system.[8] They facilitated establishment of protocols for producing lung organoids with enhanced cellular complexity through co-culturing various cell types to investigate their interactions. Another current limitation of lung organoids is their insufficient differentiation and maturation compared to native lung tissues, potentially limiting their functionality. To address this limitation, researchers have been applying bioengineering technologies, such as air-liquid interface (ALI), microfluidic chips, functional hydrogels, and co-culture methods, to lung organoid engineering. While lung organoids have not yet reached the level of the actual lung tissue, ongoing efforts to bridge this gap are promising and hold the potential to yield more physiologically relevant models for a deeper understanding of human lung biology and respiratory diseases.

Organoids have emerged as a useful tool for establishing in vitro disease models,[9–11] improving in our understanding of pulmonary diseases. With the advent of lung organoids and several bioengineering technologies, meaningful studies have been conducted rapidly. These models enable close observation of cellular interactions and disease phenotypes, offering valuable insights into the underlying pathophysiology. Moreover, they have been applied to drug screening and evaluation, providing potential treatments with higher accuracy and physiological relevance. In this review, we overview the physiological properties and cellular composition of the respiratory system. Subsequently, we describe various approaches for production of lung organoids and strategies to enhance their complexity and maturity. Lastly, we showcase the applications of lung organoids in disease modeling, including respiratory infection and pulmonary fibrosis.

## Physiological properties and cellular components of the respiratory system

The respiratory system, responsible for breathing, exhibits distinct physiological characteristics and cellular components throughout its various regions (Figure 1). As air enters the lower respiratory tract, it passes through the conducting airway parts, including the trachea, bronchi, and bronchioles, before reaching the respiratory functional unit known as the alveoli. Central to the efficient functioning of each structure are the specialized epithelial cells, which play a pivotal role in supporting their respective physiological functions.[12]

### *Conducting airway*

The conducting airway parts of the respiratory system are lined with a variety of epithelial cells, primarily including basal cells, club cells, ciliated cells, and goblet cells. They work harmoniously to facilitate the passage of inhaled air, regulate heat and moisture exchange, and efficiently eliminate foreign particles that may come along with the air.[13] It is important to note that the ratio of these cells and detailed structure vary depending on their location. The trachea, positioned at the entrance of conducting airway parts, is a tubular structure that serves as a conduit between the larynx and the lungs. One of the key structural elements is the presence of ring-shaped cartilage tissue, specifically hyaline cartilage, which imparts remarkable elasticity and stability to the trachea.[14,15] The bronchi are also tubular structures that extend from the trachea and divide into the left and right lungs. As they penetrate deeper into the lungs, they undergo a branching pattern, creating a complex network of airways. This branching arrangement gradually become thinner as they go inside the lungs, and those with a diameter of less than 1 mm are called bronchioles, which are directly connected to the alveoli. Unlike trachea or bronchi, bronchioles do not have cartilage tissue, but instead, they consist of smooth muscle that enables them to perform expansion and contraction movements.

Basal cells are considered lung progenitor cells with multipotency that can regenerate the bronchial epithelium in the event of injury.[16] These cells are found in both bronchi and bronchioles, although their distribution decreases as the bronchi become thinner. Basal cells constitute about 30% of the epithelial population in the largest bronchi with a diameter greater than 4 mm and about 6% in the smallest bronchi with a diameter less than 0.5 mm.[17] Club cells, also known as the Clara cells, are non-ciliated and non-mucous cells and are located primarily on the distal side of airway epithelium. This spatial distribution indicates that the ratio of club cells increases while the ratio of basal cells decreases toward distal regions of the airways. Club cells have secretory functions that contribute to detoxifying the inhaled air and reducing potential damage to the respiratory system.[18] Other bronchial epithelial cells act as direct physical and immune barriers against external unpurified air containing various particles. Cilia made from ciliated cells, which occupy the majority of bronchial epithelial cells, filter substances through beating, and mucus secreted from goblet cells captures these external factors and exports them to the outside.[19] Furthermore, the epithelial cells secrete soluble factors such as cytokines and chemokine, which play a crucial role in activating and recruiting immune cells, stimulating them to mount some responses against the invading particles.[20] As mentioned above, the advanced genomic analyses including scRNA-seq and spatial transcriptomics have revealed previously unknown cell types and their unique characteristics. One

such discovery is the identification of pulmonary ionocytes, which are characterized by expressing *FOXI1* and cystic fibrosis transmembrane conductance regulator *(CFTR)*.[21] In this study, the researchers unveiled that these *CFTR*-rich ionocytes could control luminal pH and be associated with the pathology of cystic fibrosis.[21] In addition, several cell types whose existence or roles are still unclear in the airway, such as pulmonary neuroendocrine cells, tuft cells, hillock cells, and microfold cells, could be detected, and their potential involvement in the immune responses has been investigated.[22–24]

### *Alveoli*

Gas exchange of passed air carries out between the alveoli, the tiny air sacs, and the capillaries surrounding them. As blood is perfused from the pulmonary artery to the pulmonary vein, blood undergoes oxygenation through gas exchange process, facilitating the distribution of oxygen-rich blood throughout the entire body.[25] The air sacs, located at the distal end of the airway, showcase a grape-like configuration with bundled alveoli clusters. This unique structure grants the alveoli a significantly large surface area relative to their volume. The expansive surface area of the alveoli is essential as it determines the capacity of the lungs to handle respiratory gas exchange and this architectural design demonstrates remarkable efficiency in maximizing gas exchange.

Within process of gas exchange, the roles of alveolar epithelial cells are crucial. The alveolar epithelial type I cells (AEC1) are the specialized epithelial cells responsible for facilitating gas exchange within the alveoli. Occupying approximately 96% of the alveolar surface, these cells form an exceedingly thin air-blood barrier, enabling efficient gas exchange.[26] On the other hand, alveolar epithelial type II cells (AEC2) possess a cuboidal morphology and are involved in the function of surfactant secretion for reducing surface tension.[27] This function is supported by an organelle known as the lamellar body, which plays a role in the synthesis and storage of surfactants. Lamellar body has a structure composed of concentric stacked layers and exists in a form combined with the cell membrane to store and release pulmonary surfactant proteins and phospholipids outside the AEC2 membrane.[28,29] Notably, the plasticity of alveolar cells is important in lung development and regeneration. AEC2 display progenitor-like characteristics, enabling them to undergo trans-differentiation into AEC1 in response to damage or during the process of regeneration.[30] The cell types comprising the alveoli are closely and firmly connected through tight junctions, forming a barrier that separates the interior and exterior of the alveoli.[31] Due to the advances in sequencing technology, studies have been conducted focusing on interactions with the surrounding systems such as capillary vessel or distal airway part.[32,33] In addition, other recent study newly characterized alveolar type 0 cells expressing *SFTPB*, *SFTPC*, and *SCGB3A2*, which are in transient state before AEC2 differentiates into AEC1 or secretory cells.[34]

## Generation and differentiation of lung organoids

Lung organoids, 3D miniaturized models of the lung, have been developed using diverse stem cells and progenitor cells, as they possess the ability to differentiate into specific cell types and self-assemble (Figure 1).[35] The complex clusters of organ-specific cells in lung organoids are created through co-culture or differentiation, resulting in a 3D structure that faithfully mimics the interactions of in vivo cell responses in lung. Generally, lung organoids are generated using two types of stem cells due to their differentiation capacity: multipotent adult stem cells (ASCs) and pluripotent stem cells (PSCs).

### *Adult stem cell (ASC)-derived lung organoids*

The versatility and potential of ASCs have been demonstrated to generate complete and highly complex lung organoids that represent different regions of the lung. ASCs obtained from lung tissue possess the self-renewal capabilities, enabling them to sustain their population over time, which makes them an ideal cell source for forming organoids. They are a population of relatively undifferentiated cells residing amidst differentiated cells in lung tissues, and their differentiation potential is limited to cell lineages present in the lung tissue. Obtained from the lung tissue biopsy, ASCs are dissociated into single cells and isolated using cell sorting to maximize the selection of the desired cell types. The concept of lung organoids was first introduced in 1987, involving the culture of cells from mouse fetus lung tissue using the medium/air interface approach on a membrane filter, resulting in the development of organoids equipped with alveolar-like lumen and basal lamina.[36] Research on sphere-forming, a process related to the culture of organoids, has been steadily increasing in the lungs and other organs. In a study conducted in 2009, human bronchospheres were cultured using basal cells, derived from human bronchial tissue and sorted based on $ITGA6^+$ $NGFR^+$ expression.[37] This study revealed the presence of $KRT14^+$ $P63^+$ basal cells located at the periphery of $KRT8^+$ luminal cells and ciliated cell, demonstrating the regenerative and multipotent characteristics of basal cells in the bronchial epithelium.[37] Another study explored the mechanism of metaplasia, where basal cells transformed into other cell types within bronchospheres derived from human basal cells.[38] Goblet cells and ciliated cells differentiated from basal cells were observed, alongside the presence of basal cells within bronchospheres.[38] This study also found that external stimuli and

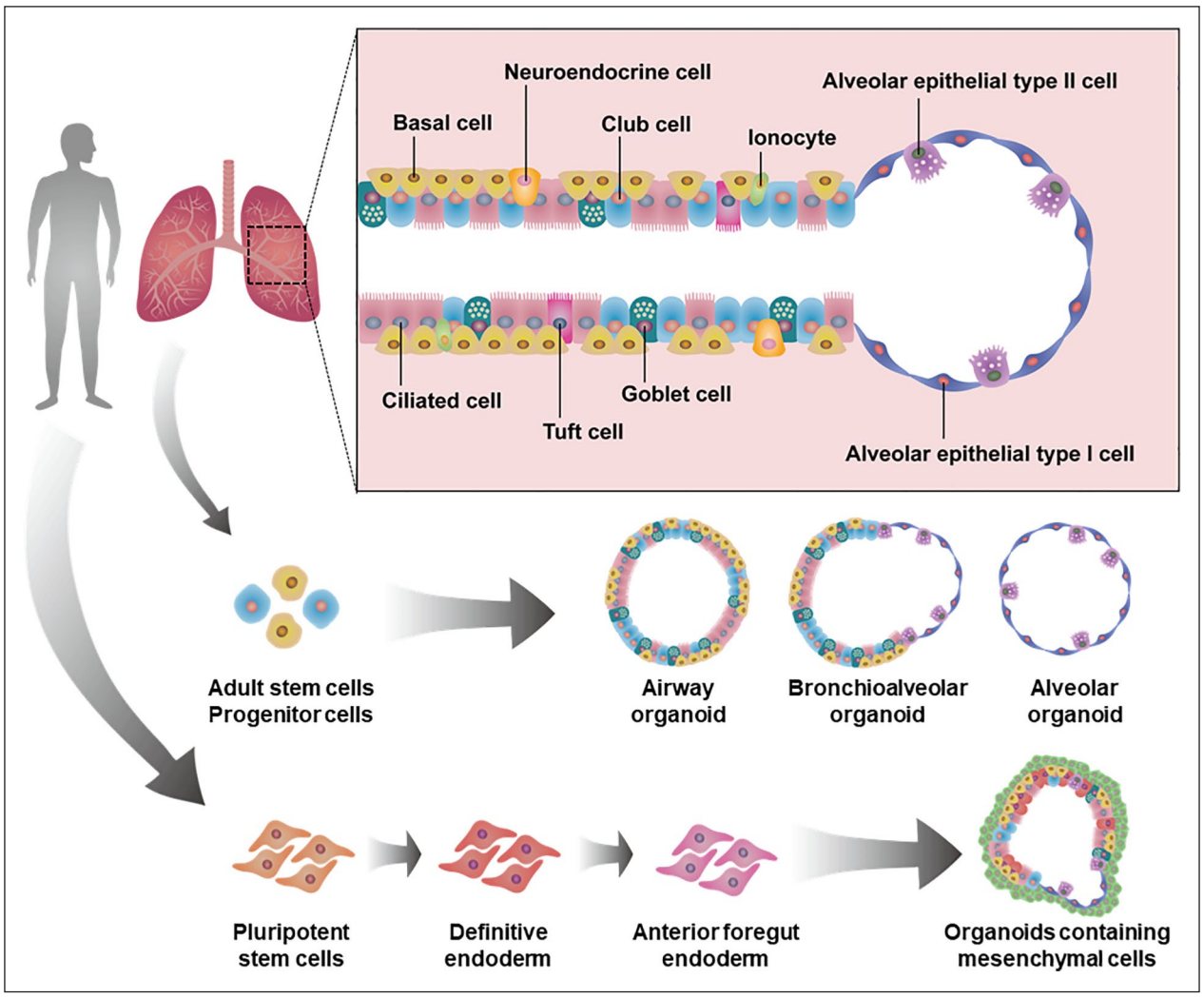


**Figure 1.** Development of lung organoids simulating the cellular components of the human respiratory system. Various epithelial cells known to exist in human conducting airway and alveoli are illustrated. Progenitor cells and adult stem cells within lung tissue can be used to generate airway organoid (bronchosphere), bronchioalveolar organoid, and alveolar organoid (alveolosphere). Pluripotent stem cells can differentiate into lung organoids containing mesenchymal cells through the stages of definitive endoderm and anterior foregut endoderm.

Notch signaling regulation were associated with the metaplasia process, specifically in the transformation of basal cells into goblet cells.[38] AEC2, known for their self-renewal and differentiation capacity, serve as alveolar progenitor cells, making them a suitable source for building alveolospheres. Particularly, surfactant protein C (SFTPC)-expressing AEC2 exhibit long-term stemness when cultured in a 3D environment, and can grow as self-renewing alveolosphere which also contains $HOPX^{+}$ AEC1.[39] The alveolospheres have been applied as a platform in various fields to study the physiology of alveolar cells.[40–43] Separately, ASC-derived lung organoids containing both proximal and distal lung epithelial cells (AEC1, AEC2, basal cell, ciliated cell, goblet cell, and club cell) were developed and utilized for COVID-19 infection modeling.[44]

## *Pluripotent stem cell (PSC)-derived lung organoids*

Lung organoids could be generated in a similar way to the process of fetal lung development using PSCs, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Particularly, organoids derived from PSCs are formed by converting 2D cells to 3D clusters with various methods. One approach involves isolating undifferentiated PSCs into embryoid bodies (EB) or partially differentiated PSCs into aggregates, and further differentiation into specific germ layers that mimic the gastrulation process during early embryo development.[45–48] Another method induces the spontaneous aggregation of cells during the differentiation process into anterior foregut endoderm (AFE).[49–51] In this approach, clumps are naturally

formed and isolated from the cell sheet when the differentiation process is carried out appropriately. By encapsulating these clumps in an extracellular matrix (ECM), they become more mature, and the resulting organoids exhibit a high degree of self-organization.

The generation of PSC-derived organoids necessitates a comprehensive understanding of each stage of lung development in an embryo. During embryonic development, the lungs originate from the endoderm, one of the three germ layers, specifically from the ventral side of the AFE. In this respect, PSCs in the undifferentiated stage are first directed to differentiate into definitive endoderm (DE), and then further induced into AFE. The differentiation protocol of PSCs to generate AFE was initially established in a 2D culture environment. In 2005, D'Amour et al.[52] reported the differentiation of PSCs into DE through activin A and serum gradient control, with high nodal signaling during gastrulation as a key condition for DE differentiation.[53] Subsequently, Green et al.[54] proposed a protocol for further differentiation into the AFE stage by simultaneously inhibiting transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) signaling, thereby taking a step closer to the possibility of reproducing lung cells from PSCs. Since then, studies have been actively conducted to make spheroids or organoids through 3D culture by embedding the cells in ECM hydrogels after the AFE stage or by continuously proceeding differentiation from the EBs. In 2015, a study reported the generation of ventral-anterior foregut spheroids, which were then cultured as human lung organoids through the regulation of fibroblast growth factor (FGF) and hedgehog signaling pathways.[50] These organoids possessed both epithelial and mesenchymal characteristics similar to human fetal lungs. As such, PSC-derived organoids mainly recapitulate developmental process, and they are mostly in a fetus-like immature state.

## *Lung organoids with cellular niches*

Despite powerful advantages of lung organoids, they have limitations, particularly in achieving maturity, functionality, and complexity, as mentioned above. Lung organoids, especially derived from PSCs, often exhibit a lower level of cell differentiation, maturation, and function compared to actual organs. To effectively function as a respiratory system, the lung organoids require incorporation of surrounding cells. In this point of view, several studies have generated lung organoids through the co-culture of different cell types including endothelial cells (ECs), mesenchymal cells, and immune cells. Researchers have employed several approaches to co-culture other niche cells with lung organoids.[55] One is mixing fully differentiated niche cells with lung epithelial cells and culturing them together as a single organoid, and the other is co-culturing niche cells with pre-formed lung organoids.

## *Endothelial cells (ECs)*

The vascularization of lung organoids is critically important in enhancing the development and maturation of organoids.[56,57] Alveolar capillaries are composed of two distinct cell types: lung-specific aerocyte capillary (aCap) cells responsible for gas exchange and general capillary (gCap) cells that serve as the stem cells for capillaries.[32] Alveolar cells are closely aligned with microvasculature, forming an air-blood barrier that facilitates efficient gas exchange. The alveoli are composed of subunits surrounded by dense capillaries, and the endothelium of the capillaries is in direct contact with the alveolar epithelium through the connective tissue layer.[58] During lung development in the embryo, pulmonary ECs undergo co-differentiation with lung tissue.[59] In the embryonic stage, primary lung lobes start to develop, and vascular endothelial growth factor (VEGF) is actively expressed in the surrounding mesenchyme, initiating the formation of alveolar capillary network.[60] Given these findings, ECs consider essential cellular niches for development of functional lung organoids.

Therefore, incorporation of ECs into lung organoids has been actively tested. In a recent study, alveolar epithelial cells, lung fibroblasts, and ECs were combined to generate organoids, named human fluorescent lung organoid (hFLO).[61] The resulting hFLOs have airspace-like lumens made of lung epithelial cells as well as branching and perfusable vasculature. Another study also showed the formation of lung organoids with vascular structures by co-culturing the bronchial epithelial cells, lung fibroblasts, and microvascular lung ECs.[62] These organoids exhibited a more flattened and extended morphology, indicating the epithelial-mesenchymal orientation during airway branching morphogenesis. Ramamoorthy et al.[63] developed multicellular lung organoid called primitive lung-in-a-dish (PLiD) by co-culturing lung epithelial cells, lung fibroblasts, lymphatic ECs, and umbilical vein ECs. PLiD can replicate the lung microenvironment, including air sac formation and surfactant protein production. Introduction of cancer cells into PLiD recapitulated the characteristics of metastatic lung tumors. In another study, Wilkinson et al.[64] co-cultured human umbilical vein ECs, small airway epithelial cells, and lung fibroblasts with collagen-functionalized alginate beads in a rotational bioreactor. Using this co-culture method, the researchers constructed multicellular 3D lung organoids expressing diverse cellular markers such as CD31, pan-cytokeratin, and vimentin.

## *Mesenchymal cells*

The lung mesenchyme provides the structural support to lung tissue and is instrumental in the differentiation during the development stage.[65] It comprises various cell types, including mesothelial cells, lymphatic cells, pericytes,

fibroblasts, and smooth muscle cells.[66] The lung mesenchymal cells are directly involved in epithelium recovery during injury repair and simultaneously differentiate into cells constituting lung epithelium, such as club cells, ciliated cells, and goblet cells.[67] In particular, the role of mesenchyme is crucial in the proliferation of alveolar epithelium. Mesenchymal cells provide tensile force, ECM proteins, and paracrine cues, known to be important for maintaining the differentiation potential of AEC2 through niches created by epithelial-mesenchymal interactions.[39,68,69]

For this reason, in several studies differentiating lung organoids, fibroblasts are often co-cultured to induce stable maintenance of AEC2 in alveolar organoids. Yamamoto et al.[70] proposed alveolar organoids embedded in ECM hydrogel with fetal lung fibroblasts and confirmed that fibroblasts are beneficial for the expansion of $SFTPC^+$ alveolar organoids and the improvement of AEC2 stemness. Tamai et al.[71] generated alveolar organoids using iPSC-derived $NKX2\text{-}1^+$ lung progenitor cells and iPSC-derived mesenchymal cells (iMES). iMES effectively induces $SFTPC^+$ $EPCAM^+$ AEC2 through Rspondin-2 and Rspondin-3 expression, suggesting a link between epithelial-mesenchymal interaction and lung organogenesis. The applicability of this in vitro model was demonstrated by conducting SARS-CoV-2 infection research. Leeman et al.[72] co-cultured mesenchymal stem cells (MSCs) with lung progenitor organoids. This co-culture method increased alveolar differentiation and decreased self-renewal in lung progenitor organoids. The researchers suggested that the effect of MSC-secreted factors, such as thrombospondin-1 (TSP1) and matrix metallopeptidase-9 (MMP-9), may be involved in lung progenitor cell function and alveolar differentiation.

### *Immune cells*

Maintaining proper immunity in the lung is important due to its continuous exposure to external air containing invisible fine particles, pathogens, allergens, and aerosols. In this regard, the interaction between immune cells and lung epithelial cells is an essential factor for the implementation of a precise disease environment. Both circulating immune cells and lung-specific immune cells coexist to maintain immune homeostasis in the lung. Innate lymphoid cells are innate immune cells responsible for maintaining tissue homeostasis in the lung and are important for defense and tissue remodeling. Meanwhile, natural killer cells perform cytolytic functions, and dendritic cells play a crucial role in initiating immune responses by presenting antigens encountered throughout the respiratory system.[73–75]

Lung-resident immunity serves as the first line of defense against threats from the external environment. Macrophages are involved in various physiological functions such as homeostasis, metabolic function, waste clearing, and tissue repair.[76] They have plasticity that can change into different phenotypes with specific functions. Alveolar macrophages (AMs) and interstitial macrophages are found in the lung, and AMs account for the majority. AMs reside in the alveolar airspace and can migrate through the pores of Kohn that connects the alveoli.[77,78] Vazquez-Armendariz et al.[79] generated bronchioalveolar lung organoids having both bronchiolar-like and alveolar-like structures using isolated bronchioalveolar stem cells, and then microinjected yolk sac-derived AMs into the organoids. Through this study, they confirmed that co-culture with AMs increased the proportion of terminally differentiated epithelial cells by upregulating *Neat1* and *Cypf2* gene expressions associated with lung epithelium maturation. Additionally, this model was applied to study macrophage-epithelium crosstalk, demonstrating the downregulation of *Ccl4*, *Il6*, and *Il8* gene expressions associated with inflammation-related stress responses. Heo and Hong[80] and Heo et al.[81] differentiated macrophages and AECs from hPSCs, dissociated them into single cells, and cultured them at a ratio of 1:5 to generate a single organoid through forced aggregation. Then, they established a pulmonary fibrosis model using TGF-β1 to induce inflammation and fibrotic changes, and drug efficacy testing was performed to demonstrate the efficacy of co-culture models. Seo et al.[82] generated human iPSC-derived macrophages (iMACs) and alveolar organoids, and then co-cultured them by injecting iMACs into alveolar organoids. These iMACs in alveolar organoids were able to exist for 14 days, and the expression of genes like *IL-1*β and *TNF*-α increased under lipopolysaccharide (LPS)-induced inflammatory conditions. This study also confirmed an elevation in proinflammatory cytokine secretion, including monocyte chemoattractant protein-1 (MCP-1), osteopontin (OPN), interleukin-8 (IL-8), and macrophage inflammatory protein-1β (MIP-1β).

## Advanced organoid culture platforms

Supporting the growth and maturation of lung organoids requires sophisticated and physiologically tailored culture methods. The scientists in organoid research field have recognized the limitations of the traditional culture systems and have tried advancing the organoid culture environments and platforms.[83] These approaches include the identification of culture medium compositions that can more precisely mimic the in vivo conditions necessary for organoid growth and development. Moreover, the intervention of biomaterials, including hydrogels and scaffolds, and advanced culture systems, including ALI and microfluidics, has improved culture environments to recapitulate the complexity and architecture of native lung tissues and their niches (Figure 2).[84] These advancements offer researchers valuable tools to study organogenesis and disease mechanisms, perform drug screening, and develop personalized medicine.

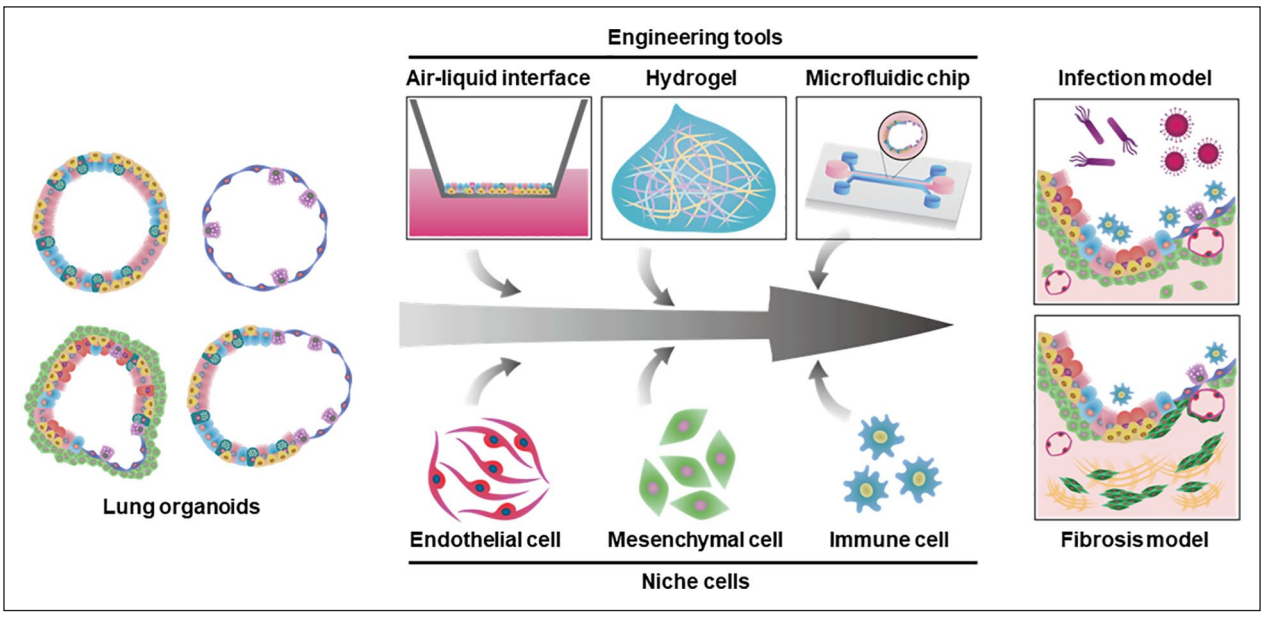


**Figure 2.** The engineering platforms and cellular niches for culturing lung organoids to model the pulmonary infection and fibrosis. The organoid culture platforms, such as air-liquid interface (ALI), hydrogel, and microfluidic chip, have been employed in a combination with various niche cells like endothelial cells, mesenchymal cells, and immune cells. These engineered lung organoid models can be used for studying pulmonary infection and fibrosis.

## *Air-liquid interface (ALI)*

The ALI system is a widely utilized approach in lung research as it effectively simulates the physiological and spatial characteristics of the actual lung.[85] In the lung, the apical surface of the epithelium is exposed to air while the basal surface is in contact with submucosal region. The basic principle of the ALI system involves placing the basolateral side of cells in contact with culture medium (liquid) and their apical side with air, which has been applied to lung epithelial cell culture.[86] Through ALI culture, human airway epithelial cells can undergo differentiation into pseudostratified epithelium, characterized by the presence of tight junctions, cilia, and mucus.[87,88] The ALI system enhances integrity in lung epithelial monolayers and increases surfactant secretion, surpassing the efficiency of conventional immersed conditions.[89] This method provides a more tailored model for co-culturing with diverse lung component cells. Importantly, these improved features make it an optimal platform for studying host-pathogen interactions in the context of lung biology.

Thus, ALI culture has been often used as a method for effectively differentiating lung organoids, offering functional and structural mimicry (Figure 3(a) and (b)).[90–92] When lung-specific cells were embedded in ECM hydrogel on a transwell and cultured using ALI method, the formed organoids showed more complex morphology containing cystic and branching structures than organoids cultured using conventional submerge culture method.[91] Furthermore, several studies established ALI-based infection models with dissociated lung organoids, which served as advanced infectious disease models with complex cell compositions containing basal cells, alveolar cells, and even neuroendocrine cells, and functional ciliary movements (Figure 3(c)).[93–95] This approach enables researchers to investigate various aspects of infection and study the interactions between pathogens and different cell types found in the lung organoids.

## *Microfluidic chip*

Microfluidic chip facilitates the provision of tissue-mimetic physiological environments and stimulation for organoids, such as vascular network, ALI, mechanical strain, and fluid flow (Figure 4).[96–99] Lung organoid culture system adopting microfluidics facilitates the seamless integration of continuous perfusion with culture media, drugs, or various factors, thereby effectively replicating fluid-induced shear stresses and emulating the physiological functions of the lungs in vivo.[89,100,105] Because of these characteristics, microfluidic system offers advantages in replicating the alveolar-capillary interface, biomechanical activity, and inherent biological functions of the lungs. Consequently, lung-on-a-chip, featuring micro-engineered microfluidics with multilayered structures, is increasingly applied in pharmacotherapeutic research, including preclinical models and disease models. Several lung-on-a-chip prototypes based on microfluidic chip are specifically designed to replicate the physical changes that occur in

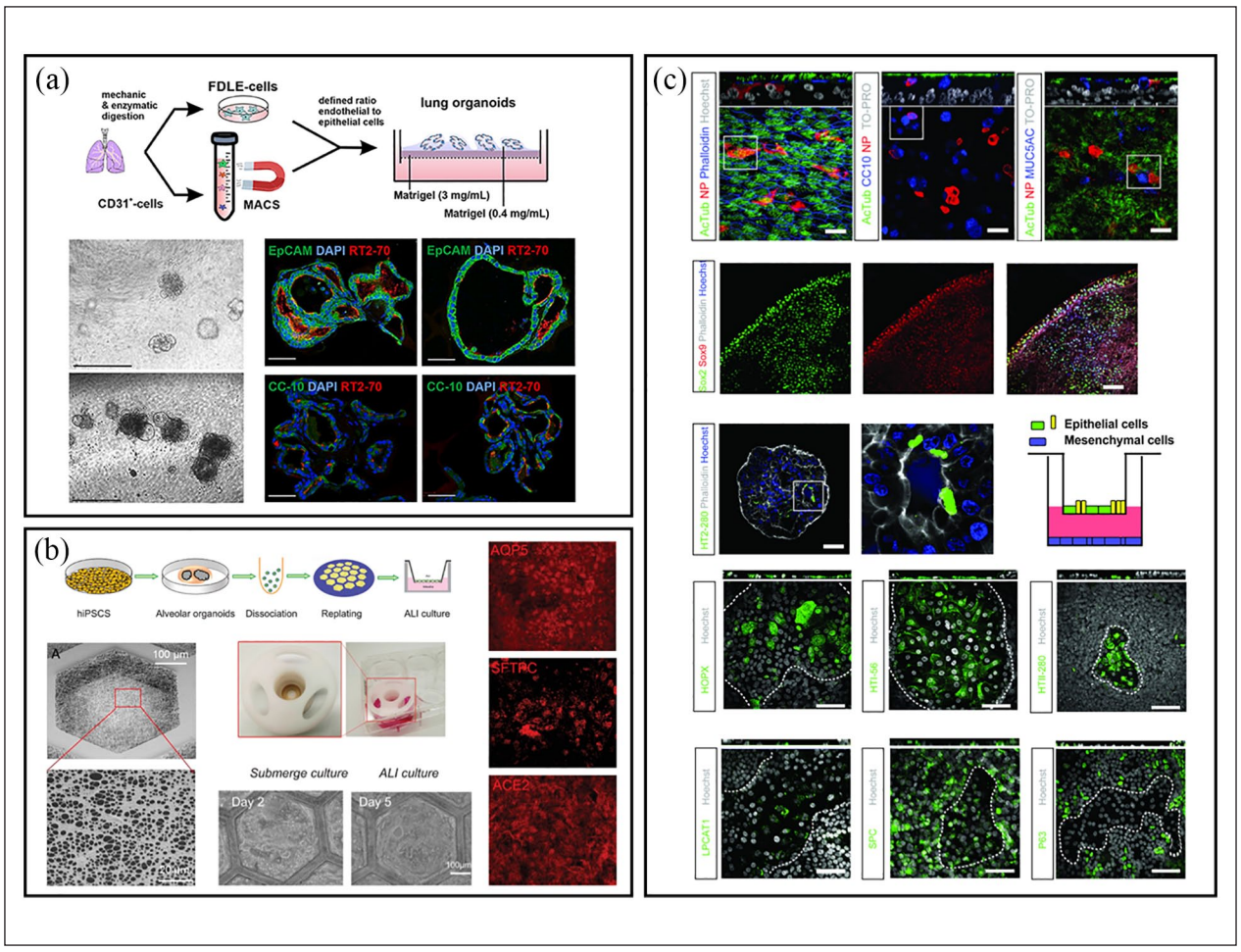


**Figure 3.** Lung organoid culture platforms based on air-liquid interface (ALI): (a) lung organoids cultured on permeable inserts exposed to air exhibited cystic and branched morphologies with lung epithelial markers (CC-10, EpCAM, and RT2-70). The images are reproduced from Laube et al.[91] with a permission from publisher, (b) alveolar organoids cultured on ALI using artificial basement membrane showed high expression of alveolar cell specific proteins and tight junction. The images are reproduced from He et al.[92] with a permission from publisher, and (c) the bronchioalveolar ALI system, established by seeding fetal lung bud tip organoids onto a transwell membrane and co-culturing mesenchymal cells on the bottom side of a well plate. This system increased the susceptibility to SARS-CoV-2 infection in AEC2 due to higher expression of the TMPRSS2, a pivotal factor in SARS-CoV-2 infection. The images are reproduced from Lamers et al.[93] with a permission from publisher.

lung tissue during respiration.[101,102] In these lung-on-a-chip systems, alveolar epithelial cells are cultured in the upper channel, while ECs are cultured in the lower channel. To mimic the alveolar-capillary barrier and create ALI, air is passed through the upper channel where the epithelial cells are located. For simulating the mechanical strain and stretching that occur during breathing, a vacuum is applied to flexible chambers located on both sides of the chip. These mechanical forces can replicate the physiological and physical changes that take place within the lungs. By integrating these dynamic features, lung-on-a-chip technologies provide a valuable platform for studying the complex interactions of lung cells and their responses to mechanical cues.

The lung-on-a-chip systems, which mimic the structural and physiological features of the lung tissues, can be applied to investigate the response of lung cells to external factors and invading pathogens. For instance, Cao et al.[106] developed a 3D alveolus-on-a-chip simulating the alveolus-capillary barrier, proposing a platform to test the pathogenesis of SARS-CoV-2 and antibody response against the virus. Baptista et al.[103] constructed an ALI lung-on-a-chip model in which alveolar epithelium was cultured for more than 14 days using the microcurved membrane that mimics the structural characteristics of alveoli (Figure 4(a)). They proposed an in vitro model capable of reproducing the interalveolar septum-like interspace through co-culture with ECs. Nof et al.[107] introduced an airways-on-chip platform designed to replicate viral infection and transmission across the respiratory system, spanning from nasal passages to bronchial airways and ending at pulmonary acini, by employing a microfluidic chip and 3D printing

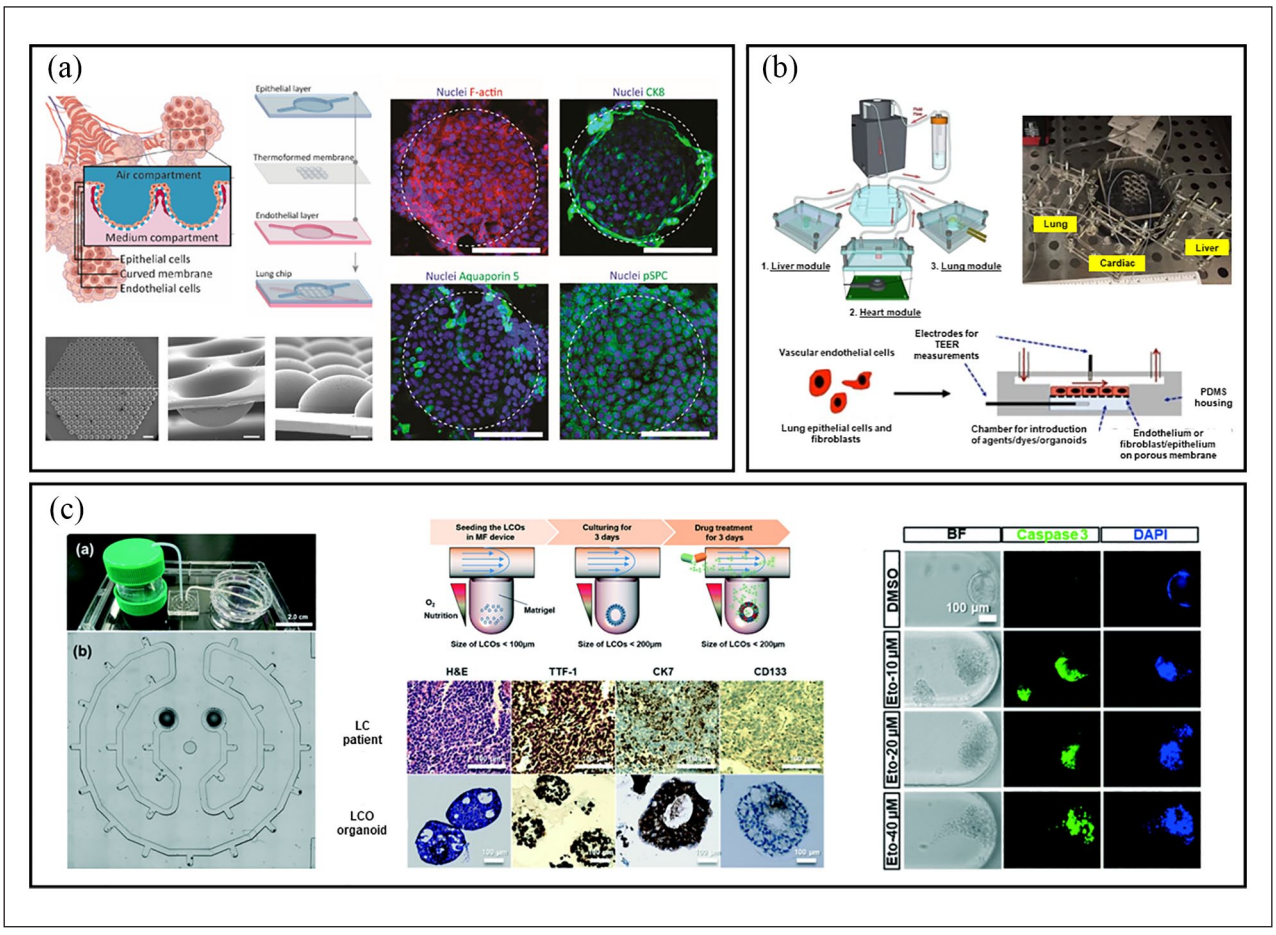


**Figure 4.** Lung organoid culture platforms based on microfluidic devices: (a) establishment of 3D lung-on-a-chip model with alveoli-like curved microwell structure and lung epithelial cell monolayer. The microwell retained a complete lining of the epithelial layer, and the expression of epithelial marker (CK8) and AEC2 marker (pro-SPC) was observed across the entire culture area. The images are reproduced from Baptista et al.[103] with a permission from publisher, (b) multi-organ-on-a-chip platform to recapitulate multi-tissue interactions among lung, heart, and liver organoids. The images are reproduced from Skardal et al.[104] with a permission from publisher, and (c) microfluidic devices with 3D lung cancer organoids used for testing the sensitivity of anti-cancer drugs. Cleaved caspase 3-positive cells were detected in lung cancer organoids treated for 48 h with anti-cancer drugs (cisplatin and etoposide), indicating apoptosis of cancer cells in a concentration-dependent manner. The images are reproduced from Jung et al.[105] with a permission from publisher.

techniques. They validated elevated levels of inflammatory cytokines induced by SARS-CoV-2 virus-laden airflow within each segment of the respiratory system. This platform effectively established a multi-compartment environment mirroring the cellular intricacy of the respiratory system.

However, these platforms do not recapitulate the morphological features of 3D organoids because they employ cell monolayer formation. In addition, organoid models outperform in vitro microfluidic chip-based cell models in achieving high-throughput culture and analysis. Organoids can be generated in large quantities, which represent a substantial number of individual models, whereas multi-compartment cell culture in microfluidic chip is complicated to secure a large number of individual models. Accordingly, organoid models enable simultaneous processing and analyses of a larger number of the samples than microfluidic cell models, leading to an increased throughput in disease modeling and drug testing.[108–110] Furthermore, recent advancements in imaging, automation, and screening methodologies facilitate the handling and analysis of the large numbers of organoids in a high-throughput manner.[111–113] Given that microfluidic systems offer distinct benefits, including precise control of biochemical and biophysical cues, the ability to mimic specific cellular behaviors, and reproducibility of the lung microenvironment, the merging of organoids and microfluidic system to capitalize on their respective strengths can provide high-throughput models with high fidelity and accuracy in the context of organoid-on-a-chip. Therefore, organoid-on-a-chip platform has been studied with microfluidic devices loaded with organoids.[114–116] This innovative platform can induce

vascularization of organoids through the fluidic shear stress and pre-vascular network within chip, thereby enhancing organoid growth and inhibiting the apoptosis of cells inside the organoids.[117–119] Furthermore, it can compartmentalize different types of organoids to investigate multi-organ interactions. Skardal et al.[104] presented an integrated organoid-on-a-chip model, in which lung, heart, and liver organoids were connected through central perfusion system (Figure 4(b)). This model demonstrated inter-organ responses such as cytokine release in the three organs responding to drug treatment. Utilizing chip-based organoid culture approach, researchers can precisely control mechanical condition, biochemical factors, and perfusion, enabling more sophisticated and comprehensive organoid research.[120]

## *Hydrogel*

3D organoids generally require supportive scaffolds to maintain their complex structures. Matrigel, extracted from mouse Engelbreth-Holm-Swarm sarcoma, has served as the gold standard for organoid culture. However, Matrigel has various limitations, including batch-to-batch variation, safety issues that restrict uses for human clinical applications, and the lack of tissue-specific 3D environment, and thus there have been the studies exploring the alternative materials for organoid culture.[121,122]

The advances in 3D hydrogels have expanded their applications to organoid culture. Indeed, various types of hydrogels have been adapted for organoid culture, including hydrogels derived from natural sources such as collagen,[123,124] fibrin,[125] and alginate[126,127] as well as hydrogels fabricated from synthetic polymers like polyethylene glycol (PEG),[128–130] polyisocyanide (PIC),[131] polyacrylamide (PAAm),[132] and polyvinyl alcohol (PVA).[133] In the case of natural hydrogels, they can offer ECM-mimicking bioactive matrices replete with cell adhesion sites for supporting organoid growth.[134] For instance, alginate hydrogels were tested for culturing human airway organoids and compared with the conventional Matrigel culture.[135] This study demonstrated that airway organoids encapsulated in alginate hydrogels exhibited increases in the number of multiciliated cells and MUC5AC$^+$ goblet cells producing mucus, while such increases were not observed in Matrigel culture. Synthetic polymer hydrogels have been examined for organoid culture owing to their advantages of ease of fabrication and the ability to control desired mechanical properties.[136] Furthermore, synthetic hydrogels can be readily functionalized with bioactive moieties, such as growth factors and adhesion peptides like fibronectin-derived peptide sequence (Arg-Gly-Asp; RGD), allowing the researchers to tailor the microenvironments to promote organoid growth and differentiation.[128,130,133] One commonly used synthetic hydrogel for organoid culture is PEG-based hydrogel, which is highly biocompatible owing to its low toxicity and minimal inflammatory responses.[130,137,138] Cruz-Acuna et al.[130] tested a PEG hydrogel prepared with the four-arm PEG macromer with maleimide groups at each terminus (PEG-4MAL) and functionalized with RGD for human lung organoid culture (Figure 5(a)). Lung organoids grown in the engineered PEG hydrogel exhibited high viability and underwent structured lung epithelium development, which included lumen formation through epithelial cell cyst growth and polarization during epithelial morphogenesis, accompanied by the expression of lung epithelial and basal cell markers.

Decellularized tissue-derived ECM (dECM) hydrogel has emerged as a promising alternative to the conventional matrices in organoid culture.[141–146] dECM hydrogel is highly enriched with various ECM proteins, including collagen, fibronectin, laminin, and proteoglycan, thus providing ECM networks for cell growth and differentiation.[136,147–149] The presence of tissue-specific ECM components in dECM hydrogel has also been underscored as organoids may require different ECM cues in a tissue dependent manner.[150] For instance, laminin-111 could be essential for the intestinal organoid culture and laminin-111, collagen IV, and fibronectin could play an important role in supporting the bile duct organoid culture.[151] Likewise, lung-derived dECM hydrogels have been employed to culture lung organoids, as well as lung cancer cells and lung fibroblasts.[152,153] Choi et al. utilized porcine lung dECM hydrogel to culture patient-derived lung cancer organoids by providing the native lung tissue ECM-like microenvironments.[154] The lung dECM hydrogel not only promoted the growth of lung cancer organoids, but also helped the organoids retain the histological, molecular, and genetic characteristics of the natural cancer tissue. In another recent study, Hoffman et al. represented decellularized human lung alveolar ECM hydrogel for human AEC2-derived alveolosphere culture (Figure 5(b)).[139] This study demonstrated that the alveolar ECM hydrogel maintained crucial ECM components such as fibrillar collagens (COL1 and COL3), laminins, proteoglycans, and especially fibrillin-1 (FBN1), a microfibrillar ECM protein essential for elastin formation. Culture of AEC2-derived alveolospheres in this dECM hydrogel facilitated AEC2 proliferation and upregulation of AEC2-derived transitional cell state genetic markers, which highlighted the importance of alveolar ECM in AEC2 differentiation. Kusoglu et al.[140] analyzed dECM hydrogels prepared from bovine lung tissues using four different decellularization protocols (Figure 5(c)). They revealed that the resultant hydrogels had significantly different ECM content, stiffness, and viscoelastic properties depending on the decellularization methods. Notably, the dECM hydrogel produced via treatment of peracetic acid, Triton-X-100, and sodium deoxycholate (SDC) showed low mechanical properties and poor gelation capacity, and the dECM hydrogel produced using sodium dodecyl sulfate (SDS) showed cytotoxicity and impaired cell growth. In contrast,

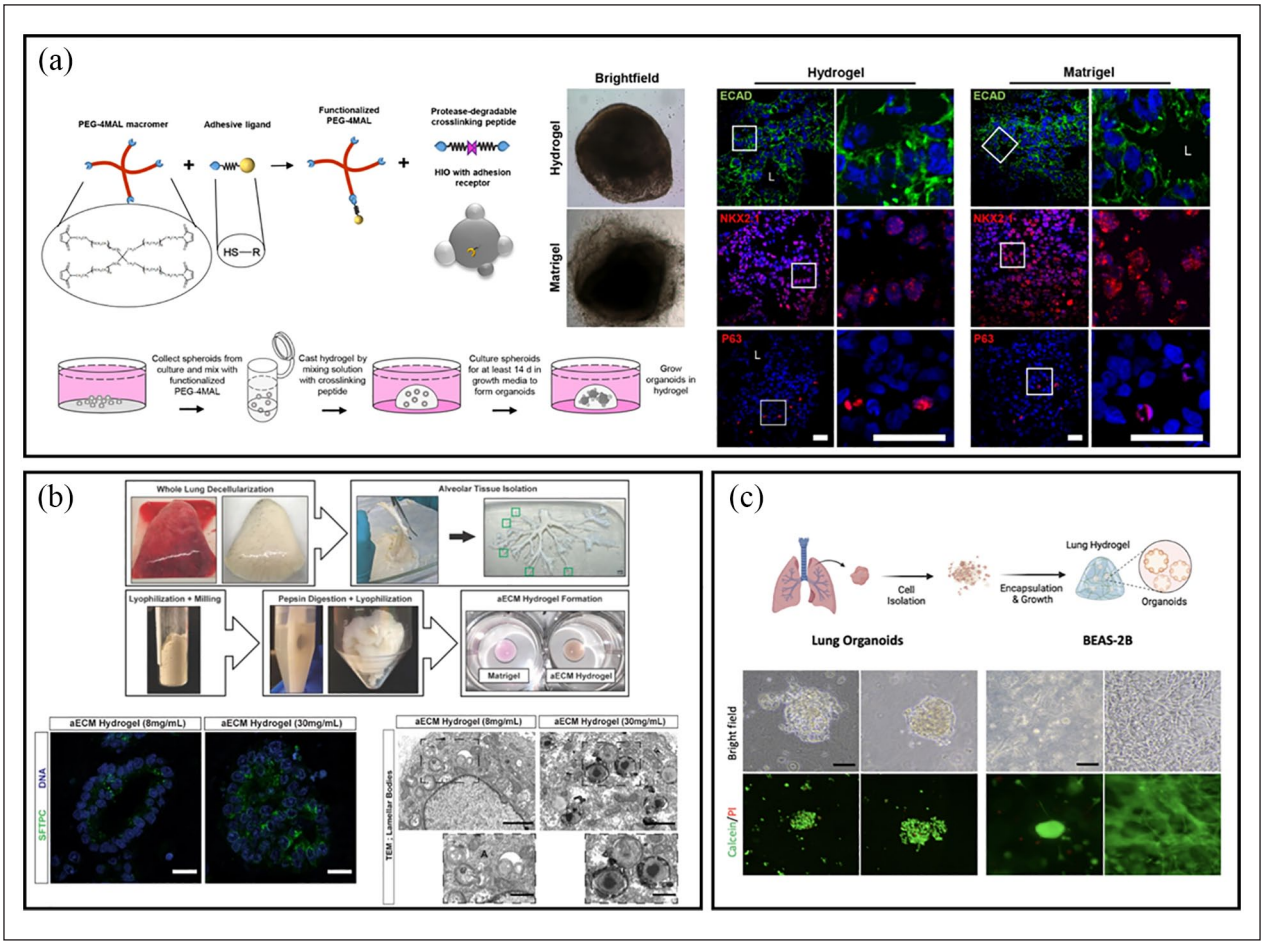


**Figure 5.** Lung organoid culture platforms based on functional hydrogels: (a) PEG-4MAL hydrogel functionalized with RGD exhibited not only the enhanced cytocompatibility, but also the potential to support lung organoids comparable to Matrigel, as evidenced by the organized expression of lung epithelium (E-cadherin) and lung-specific markers (NKX2-1 and P63). The images are reproduced from Cruz-Acuna et al.[130] with a permission from publisher, (b) decellularized human lung alveolar-enriched ECM hydrogel for culturing human AEC2-derived alveolospheres. This ECM hydrogel enhanced AEC2 proliferation and upregulated expression of AEC2-derived transitional cell state genetic markers. The images are reproduced from Hoffman et al.[139] with a permission from publisher, and (c) the expansion and growth of lung organoids in decellularized bovine lung-derived dECM hydrogel. Lung dECM hydrogels were prepared with different decellularization methods, including freeze-thaw cycles or Triton-X-100 treatment, and compared by evaluating the morphology and viability of lung organoids. The images are reproduced from Kusoglu et al.[140] with a permission from publisher.

patient-derived lung organoids cultured in two types of dECM hydrogels prepared using freeze-thaw cycles or Triton-X-100 exhibited high viability and expansion. This study underscores the pivotal role of decellularization methods in determining the characteristics and functionality of the resultant dECM hydrogels for organoid culture.

## Respiratory infection modeling

The urgency to better understand the precise mechanisms of lung diseases and develop effective treatments has driven the establishment for more accurate and advanced in vitro lung models. While research on infectious pulmonary diseases has been conducted before, the outbreak of the COVID-19 pandemic in 2019 urged a rapid increase in the research on in vitro respiratory models for investigating viral infections (Table 1).[155]

### *Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)*

SARS-CoV-2 mainly infects through the oral mucosa and lungs, and infection occurs through membrane fusion after the spike protein in the virus binds to the receptor called angiotensin-converting enzyme 2 (ACE2).[170] The researchers confirmed the expression of ACE2 on the apical side of AEC2 in lung organoids cultured from HTII-280$^{+}$ cells of adult lung tissue.[171] This finding suggests the possibility

**Table 1.** Respiratory infection modeling with lung organoids.

| Modelled disease | Origin cell | Organoid type | Cell composition | Culture platform | Features | References |
|---|---|---|---|---|---|---|
| SARS-CoV-2 | Human ASC (Fetal lung epithelial bud tips) | Lung bud tip organoid | AEC1, AEC2, basal cell | 3D gel encapsulation (Matrigel)<br>Air-liquid interface | - Bronchioalveolar ALI system<br>- Co-culture with mesenchymal cells<br>- Higher viral titer than 3D organoid model<br>- Viral capsid colocalization with HOPX+, HTII-280+, and SFTPC+ cells | Lamers et al.[93] |
| | Human ASC (Bronchial epithelial cell (NHBE)) | Bronchial organoid | Ciliated cell, basal cell | Air-liquid interface | - Higher infection efficiency<br>- High vulnerability of ciliated cells to viral infections than basal cells | Sano et al.[156] |
| | Human ASC (Airway and lung cells) | Bronchiolar and alveolar organoid | Basal cell, secretory cell, multi-ciliated cell, club cell, goblet cell | 3D gel encapsulation (Matrigel) | - Further differentiation into bronchiolar and alveolar organoid<br>- In situ expression of viral entry receptors<br>- Syncytia formation in organoid | Ekanger et al.[157] |
| | Human PSC | Lung organoid | AEC1, AEC2, fibroblast cell | 3D gel encapsulation (Matrigel) | - AEC2s particularly permissive to SARS-CoV-2 infection<br>- Robust induction of chemokines after virus infection | Han et al.[158] |
| Respiratory syncytial virus (RSV) | Human PSC | Lung organoid | Ciliated cell, goblet cell, club cell, mesenchymal cell | 3D gel encapsulation (Matrigel) | - Disruption of F-actin cytoskeleton | Harfold et al.[159] |
| | Human PSC | Lung organoid<br>Lung bud organoids | Alveolar epithelial cell, goblet cell, club cell precursor | 3D gel encapsulation (Matrigel) | - Swelling, detachment and shedding of infected cells into lumens | Chen et al.[48] and Porotto et al.[160] |
| | Human PSC | Lung organoid | Airway epithelial cell, alveolar epithelial cell | 3D gel encapsulation (Matrigel) | - Increased DNA damage and cell death when viruses treated with PM<br>- Increased stress granules in RSV-infected conditions | Choi et al.[161] |
| | Patient-derived lung tissue | Airway organoid | Basal cell, secretory cell, multi-ciliated cell, club cell, goblet cell | 3D gel encapsulation (Matrigel) | - Increased organoid cell motility via the non-structural viral NS2 protein<br>- Recruitment of neutrophils upon co-culture | Sachs et al.[162] |
| Human adenoviruses | Human PSC | Airway organoid<br>Alveolar organoid | Basal cell, ciliated cell, goblet cell, club cell<br>AEC1, AEC2 | 3D gel encapsulation (Matrigel) | - Higher infectivity of hADV-55 to basal cell and AEC2 than hADV-3<br>- Impairment of self-renewal functions post-injury<br>- Loss of cell differentiation in lungs | Zhao et al.[163] |
| Influenza A virus | Human ASC (Normal lung tissue) | Airway organoid | Ciliated cell, goblet cell, basal cell, club cell | 3D gel encapsulation (Matrigel) | - Enhanced proximal differentiation<br>- Highly human-infective H1N1pdm virus: higher titer in organoid | Zhou et al.[164] |
| | Human PSC | Alveolar type II organoid | AEC2, AEC1, lung endoderm cell | 3D gel encapsulation (Matrigel) | - Upregulated ACE2 and TMPRSS2 | Kim et al.[165] |
| Bacteria | Human ASC (Lung tissue) | Airway organoid | Basal cell, secretory cell, multi-ciliated cell | 3D gel encapsulation (Matrigel) | Mycobacteria (Mtb, Mab)<br>- Mtb; viable for up to 21 days within the organoid lumen<br>- Mab; actively replicated over 7 days<br>- Modulation of cytokines and antimicrobial peptides<br>- Inhibition of mucin expression | Iakobachvili et al.[166] |
| | Human ASC (Bronchial tissue) | Lung organoid | Basal cell, ciliated cell, goblet cell, club cell | 3D gel encapsulation (BME2) | *Cryptosporidium*<br>- Observed various epicellular stages of *C. Parvum*<br>- Formation of oocysts within lung organoids | Heo et al.[167] |
| | Human ASC (Primary airway tract epithelial cells) | Airway organoid | Multiciliated cell, goblet cell, basal cell | Air-liquid interface | *Pseudomonas aeruginosa*<br>- Upregulated quorum sensing (QS) and protein secretion systems<br>- Promoted immune evasion by QS | Tang et al.[168] |
| | Human PSC | Lung bud organoid | AEC2, AEC1, basal cell, ciliated cell, mesenchymal cell | 3D gel encapsulation (Matrigel) | *Streptococcus pneumoniae*<br>- Interactions of pneumococcus with alveolar epithelium (adherence, internalization, initial alveolar innate response of bacteria)<br>- Observation of pneumococcal cells in the lumen of the organoid branches, cell surface, and cell cytoplasm with immersion | Sempere et al.[169] |

that lung organoid could imitate the respiratory infection mechanism of SARS-CoV-2. Lung organoids infected with SARS-CoV-2 have been applied to identify differences in degeneration, reaction, and function of respiratory cells depending on the degree of infection.[44,158,172–177]

While the method of inoculating viruses into lung organoids has revealed various infection mechanisms and their effects on lung epithelial cells, further studies have been performed to develop more convenient and advanced infection platforms. Lung organoids typically grow in the apical-in orientation, where the apical side faces the interior of the organoids. The recently established apical-out organoid culture method can facilitate the interactions between the virus and organoid epithelium only with simple virus inoculation to culture medium.[178,179] Another advanced system based on ALI culture investigated the interaction of virus with lung organoids.[156] Lamers et al.[93] constructed a bronchioalveolar ALI system by seeding fetal lung bud tip organoids on a transwell membrane and co-culturing mesenchymal cells on the bottom side of a well plate. This model offers advantages for antiviral drug screening, as its viral titer is higher than that of a 3D organoid model. They infected the model with SARS-CoV-2 and confirmed that the virus successfully infects the alveolar epithelial cells by demonstrating that nucleoprotein representing the viral capsid colocalizes with $HOPX^+$, $HTII\text{-}280^+$, and $SFTPC^+$ cells. Additionally, they conducted drug testing with interferon-λ1 in this model system and found a significant inhibition of SARS-CoV-2 replication at a low concentration of drug. In another study, Sano et al.[156] constructed an ALI culture model with bronchial organoids and observed an infection efficiency 1,000 times higher than that of the organoid model. In their model, ciliated cells were infected with virus after 7 days from inoculation, whereas basal cells not only remained uninfected but also survived and underwent subsequent differentiation into ciliated cells. This observation highlights the potential of this research platform for investigating different responses of bronchial cells to SARS-CoV-2 and airway regeneration after viral infection.

### *Respiratory syncytial virus (RSV)*

While much attention has been recently given to COVID-19, other significant and perilous respiratory infections such as RSV have also been the subject of virus research. RSV is known to cause lower respiratory tract infection, particularly common in infants, and to be transmitted through contact or respiratory droplets.[180] As an acute respiratory infection with frequent seasonal outbreaks, this virus causes symptoms resembling the common cold, but it can be particularly severe in infants and the elderly. Consequently, investigation on disease mechanisms using lung organoids are actively underway for RSV.[159–162,181] Harford et al.[159] conducted a study monitoring the infection pattern after microinjecting RSV into human lung organoids. They observed that infectivity began to rise 72 h after viral infection. Furthermore, the expression of CC10, a club cell marker, increased in the infected lung organoids, while the expression of FOXJ1, a ciliated cell marker, decreased. Specifically, RSV disrupted the E-cadherin structure, and rearrangements of the cytoskeletal architecture were observed 5 days after infection. Another study compared the infection pattern of RSV and that of other respiratory viruses. Porotto et al.[160] infected human PSC-derived lung organoids with respiratory viruses such as RSV, human parainfluenza virus type 3 (HPIV3), and measles virus (MeV).[48], They showed that RSV infection shed infected cells into the lumen of lung organoids, while HPIV3 infection induced viral shedding without morphological changes and MeV infection distinctly caused syncytium formation. In a recent study, Choi et al.[161] explored the correlation between RSV infection and particulate matter (PM). When PM, which contains carcinogens and allergen floating in the urban polluted air, is continuously exposed to human, it accumulates inside the human body, causing a cytotoxic effect. The study confirmed that DNA damage and cell death increased when RSV-infected human lung organoids were treated with PM, particularly when the lungs were already under stress due to viral infection. Additionally, formation of stress granules, which did not occur when PM was treated alone, was significantly increased in RSV-infected lung organoids. Through this observation, this study suggested that exposure to PM can cause serious lung damage when the lungs are under stress such as viral infection.

### *Other viruses*

Human adenoviruses type 3 (HAdV-3) and type 55 (HAdV-55) have different infectivity and pathogenicity, but the understanding their infection mechanisms has been challenging due to lack of suitable models. In this regard, Zhao et al.[163] developed an in vitro model using human airway and alveolar organoids to infect each virus. They identified difference in the replication capacity of the two viruses and the degree of stem cell infection. Through this study, they demonstrated that Cidofovir, nucleotide analogue that acts as an antiviral drug, could repress the replication of the viruses effectively. Similarly, influenza A virus, a highly transmissible respiratory virus responsible for seasonal epidemics and occasional pandemics, has been the subject of organoid-based studies.[157,164,165] Infection of influenza A virus begins when its hemagglutinin binds to sialic acid of membrane receptors of host cells.[182] Zhou et al.[164] constructed human airway organoids with enhanced proximal differentiation, promoting the expression of ciliated cells. This enhancement resulted in increased expression of serine proteases, essential for productive influenza virus infection, thus creating a more suitable platform for analyzing viral infections.

### Bacteria

In addition to viruses, bacteria are important pathogens causing infectious lung diseases, and thus the studies infecting lung organoids with such microorganisms are emerging. Bacterial lung infections can manifest in various forms of illness, including pneumonia, bronchitis, and tuberculosis. Multiple ongoing studies are actively investigating the mechanisms underlying bacterial infections in lung organoid models. Various species of microorganisms such as mycobacteria,[166] *Cryptosporidium*,[167] *Pseudomonas aeruginosa*,[168] *Streptococcus pneumoniae*,[169] *Aspergillus fumigatus*,[183] and *Mycobacterium abscessus* (Mab)[184] are being investigated in lung organoid models. Leon-Icaza et al.[184] infected airway organoid with Mab via microinjection. The inoculated Mab entered the exponential growth phase 4 days post-infection and actively propagated over 12 days. Mab infection triggered oxidative stress within the airway organoids. The researchers then induced the pharmacological activation of antioxidant pathways by utilizing the NAD(P)H: quinone oxidoreductase 1 (NQO1) and a first line antibiotic cefoxitin, resulting in inhibition of Mab growth in the airway organoid. In another study, Sempere et al.[169] investigated interactions between lung bud organoids and *Streptococcus pneumoniae*, focusing on the effect of bacterial infection on alveolar epithelium. The researchers observed the transit of the bacteria through the organoid epithelium, an essential mechanism in bacterial infection process. They also emphasized host-pathogen interactions such as adherence to the AEC2 surface and internalization. These studies using lung organoid infection models have expanded our understanding of the bacterial infection mechanisms and host-pathogen interactions within the lung microenvironment, which contributes to development of potential therapies for infectious lung diseases.

## Pulmonary fibrosis modeling

Pulmonary fibrosis is a complicated and potentially life-threatening condition characterized by the abnormal accumulation of fibrous tissue, leading to lung scarring, thickening of lung tissues, and reduced respiratory function. As the body ages, the remodeling capability of the lungs deteriorates, which renders lungs more susceptible to fibrotic and inflammatory conditions, ultimately leading to idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD).[185,186] Aging is a critical risk factor for IPF given that regeneration failure of AEC2 caused by alveolar senescence has been identified as an important contributor to IPF.[187] Consequently, the decline in lung function becomes an inevitable part of aging process, and pulmonary fibrosis and the resultant lung failure affect a significant portion of the populations worldwide. Given the increasing prevalence of these fibrotic diseases, investigating their underlying causes and screening therapeutic agents for pulmonary fibrosis are of great importance. In this regard, lung organoid systems can substantially contribute to sophisticated lung disease modeling and mechanistic studies (Table 2).

### Idiopathic pulmonary fibrosis (IPF)

IPF is a common and aggressive lung disease, resulting from repeated lung epithelial damage. While drugs like pirfenidone and nintedanib can slow fibrosis progression, they cannot fully restore damaged lung cells.[191,196] For this reason, it is necessary to explore the pathogenesis of IPF precisely and develop fundamental treatment methods. Bleomycin, a chemotherapeutic antibiotic with antitumor activity, has been typically utilized to induce pulmonary fibrosis, inspired from the fibrosis phenomenon observed during cancer treatment. Indeed, various studies have induced pulmonary fibrosis through bleomycin treatment in animal models and lung organoids.[61,191] Alveolar organoids treated with bleomycin recapitulated the pathological features of IPF, including phenotypic changes such as epithelial cell-mediated fibroblast activation and cellular senescence.[191] Notably, researchers found that inhibiting activin receptor-like kinase 5 (ALK5) can alleviate fibrogenic changes in lung organoids and maintain the differentiation state of alveolar epithelial cells.

TGF-β is another well-known factor that induces pulmonary fibrosis. Upon treatment with TGF-β, alveolar epithelial cells undergo epithelial-mesenchymal transition (EMT), transforming epithelial into mesenchymal cells such as fibroblasts.[197] This phenotypic change contributes to the initiation and progression of fibrosis in the lung tissue. Kim et al.[192] developed a pulmonary fibrosis model by treating human PSC-derived alveolar organoids with TGF-β1. The establishment of IPF model was confirmed with increased expression of ECM, mesenchymal markers, fibroblast to myofibroblast transition (FMT) markers, and fibrotic area. Wilkinson et al.[64] employed a unique approach to fabricate fibrotic organoids by aggregating hydrogel beads seeded with either fetal lung fibroblasts or human iPSC-derived mesenchymal cells. In this experiment, the organoids treated with TGF-β1 exhibited irreversible scarring of lung tissue, a characteristic hallmark of IPF. Additionally, there was a significant increase in fibrotic phenotypes, as evident from elevated levels of collagen I and α-smooth muscle actin (α-SMA) expression in the TGF-β1-treated organoids.

IPF can also be caused by genetic factors. Using CRISPR/Cas9, Strikoudis et al.[5] generated lung organoids with genetic mutations for modeling Hermansky-Pudlak syndrome (HPS)-associated interstitial pneumonia (HPSIP) that exhibits clinical similarities to IPF. They found that introducing all HPS mutations ($HPS1^{-/-}$) induced fibrosis in the lung organoids. In addition, they

**Table 2.** Pulmonary fibrosis modeling with lung organoids.

| Modelled disease | Origin cell | Organoid type | Cell composition | Culture platform | Features | References. |
|---|---|---|---|---|---|---|
| Chronic obstructive pulmonary disease (COPD) | Patient-derived lung tissue (Primary bronchial epithelial cells) | Bronchial organoid | Cycling basal cell, basal cell, club cell, goblet cell, ciliated epithelial cell | Air-liquid interface | - Goblet cell hyperplasia<br>- Reduced ciliary beat frequency | Chan et al.[175] |
| | Human ASC (Lung tissue) | Lung organoid : airway, alveolar type | Ciliated cell, AEC2 | 3D gel encapsulation (Matrigel) | - Co-culture with human MRC-5 fetal lung fibroblasts | Wu et al.[188] |
| | Patient-derived lung tissue (Primary AEC2) | Alveolar organoid | AEC2 | 3D gel encapsulation (BME2) | - Obtained lung tissue from long-term smokers, patients with COPD and highly emphysematous lung tissue<br>- Application in breathing mimetic cyclic stretching PDMS membrane | van Riet et al.[189] |
| | Human PSC | Bronchioalveolar organoid | Goblet cell, basal cell, secretory cell, mesenchymal stromal cell, club cell, AEC1, AEC2 | Suspension culture | - Upregulated fibrosis- and inflammation-related transcripts<br>- Increased levels of NO after exposure to cigarette smoking extract<br>- Upregulated oxidative stress-related and pro-inflammatory genes | Jeong et al.[190] |
| Idiopathic pulmonary fibrosis (IPF) | Human PSC | Alveolar organoid | AEC1, AEC2 | 3D gel encapsulation (Matrigel) | - Epithelium dependent contraction<br>- Abnormal differentiation state and cellular senescence in AEC2 similar to disease | Suezawa et al.[191] |
| | Human PSC | Alveolar organoid | AEC1, AEC2, alveolar progenitor cell, mesenchymal cell | Suspension | - Increased ECM, mesenchymal, FMT marker, $\alpha$-SMA expression, and fibrotic area after TGF-$\beta$1 treatment<br>- Fibrogenic responses and collagen accumulation<br>- Ameliorated disease phenotypes by treatment of NP-011 via suppression of ERK signaling | Kim et al.[192] |
| | Human PSC | Lung organoid | Endothelial cell, epithelial cell, mesenchymal cell, AEC1, AEC2, goblet cell, club cell | Hydrogel beads aggregation | - Irreversible scarring<br>- Significant increase in fibrotic phenotype (elevated collagen I and $\alpha$-SMA expression) | Wilkinson et al.[64] |
| | Human PSC | Alveolar organoid | AEC1, AEC2, basal cell, club cell, ciliated cell | 3D gel encapsulation (Matrigel) | - Increased KRT5−/KRT17+ aberrant basaloid phenotype (IPF lung)<br>- Reduced expression of ECM and pro-fibrotic mediators by nintedanib and pirfenidone | Ptasinskiet al.[193] |
| | Human PSC | Lung organoid | Basal cell, goblet cell, ciliated cell, club cell, AEC1, AEC2 | Alginate hydrogel | - Mesenchymal outgrowth<br>- Increased vimentin expression<br>- Significantly increased SMA expression | Dye et al.[135] |
| Cystic fibrosis (CF) | Patient-derived PSC | Airway organoid | Basal cell, goblet cell, secretory cell, multiciliated cell | 3D gel encapsulation (Matrigel) | - Defect in forskolin-induced swelling<br>- Correction of disease mutation via gene editing | McCauley et al.[194] |
| | Patient-derived lung tissue | Airway organoid | Basal cell, secretory cell, multi-ciliated cell, club cell, goblet cell | 3D gel encapsulation (Matrigel) | - Assessment of CFTR function in an organoid swelling assay | Sachs et al.[162] |
| | Patient-derived nasal epithelial cell | Nasal-airway organoid | Basal cell, secretory cell, ciliated cell, club cell, goblet cell | 3D gel encapsulation (Matrigel) | - Treatment of neuregulin-1$\beta$ and interleukin-1$\beta$ for organoid culture<br>- Consistent detection of CFTR modulator responses in nasal-airway organoid | Amatngalim et al.[195] |

confirmed fibrotic changes in lung organoids characterized by increased expression of PDGRα and SMA and verified that overexpression of IL-11 affects fibrosis initiation.

### *Chronic obstructive pulmonary disease (COPD)*

COPD and IPF share common symptoms like dyspnea (shortness of breath), but their underlying causes are distinct.[198,199] IPF is characterized by the progressive scarring and stiffening of lung tissue due to unknown reasons, while COPD is primarily caused by damage to air sacs and bronchial tubes. This damage leads to reduced airflow in airway, inflammation, and increased mucus production, collectively resulting in breathing difficulties. Chan et al.[175] developed bronchial organoids from cells of COPD patients and confirmed goblet cell hyperplasia and reduced ciliary beat frequency in the COPD organoids. They also infected COPD organoids with SARS-CoV-2 and found that infectivity, inflammation, and viral replication were further increased. This research validated the effectiveness of organoid models in studying host-pathogen interactions within the context of disease. Wu et al.[188] investigated smoking-related COPD by exposing mice to cigarette smoke and performing transcriptomic analysis to identify target genes for potential drugs. Moreover, they treated human and mouse lung organoids with cigarette smoke extract, and revealed suppressed organoid formation and increased susceptibility of alveolar organoids to cigarette smoke compared to airway organoids. They also studied the therapeutic effects of two drugs, misoprostol and iloprost, by confirming organoid formation using the lung epithelial cells from mice exposed to cigarette smoke and drugs.

### *Cystic fibrosis (CF)*

CF is a genetic disease caused by mutations in the *CFTR* gene, resulting in impaired chloride transport.[200] This mutation also hinders the proper formation of mucus in the patient's organs like lungs and pancreas. The thickened mucus in the lungs causes airway obstruction and inflammation, and also increased susceptibility to respiratory infections.[201] Over time, this process leads to fibrosis and permanent the lung tissue scarring. McCauley et al.[194] generated CF airway organoid models using iPSCs obtained from CF patients and corrected the *CFTR* gene mutation using gene editing. Then, they verified the restoration of the CFTR channel function with forskolin-induced swelling assay, indicating the potential of the lung organoid as a valuable platform for disease modeling and gene therapy applications. In another study conducted in 2019, Sach et al.[162] explored CFTR modulator response in airway organoids from CF patients. In a subsequent study, Amatngalim et al.[195] converted 2D ALI models into 3D lung organoids to enhance differentiation homogeneity. Using this organoid and culture conditions containing neuregulin-1β and interleukin-1β, they successfully detected coherent CFTR modulator responses.

## Conclusions and outlooks

This review provides an overview of the advancements in lung organoid development, leveraging niche cells and bioengineering approaches, and their applications in modeling pulmonary diseases. While these advances have substantially enhanced the complexity, maturity, and functionality of lung organoids, there is still room for technological improvement.

Firstly, exploration of rare lung epithelial cells and incorporation of those cells into lung organoids are required for improving respiratory disease modeling. With the latest sequencing technology, previously unknown lung cell types and their roles in disease occurrence and progression have been unveiled.[202] For example, CFTR-rich ionocytes have been identified to have certain roles in the pathogenesis of CF,[203] underlining the presence of rare lung cell types in lung organoids for precise disease modeling. Similarly, lung organoids containing rare neuroendocrine cells have been developed through co-culture with fetal lung fibroblasts and ALI technique.[93] In this way, the studies have aimed at developing lung organoids that better recapitulate the cellular compositions of native lung epithelium and validating them with analytical technologies such as scRNA-seq and spatial transcriptomics.

Secondly, advanced cell engineering strategies are imperative to facilitate efficient co-culture of lung organoids with niche cells or other types of organoids. For instance, the cell engineering for developing multilineage organoids with improved maturation has demonstrated remarkable achievements, which include the production of assembloids by physically fusing different types of organoids or the fabrication of multi-tissue models via multilineage differentiation (Figure 6(a)).[3,55] Assembloid technique has been primarily applied in brain organoid studies,[204–208] and human hepato-biliary-pancreatic organoids were also developed through assembling anterior gut spheroid and posterior gut spheroid.[209] Additionally, simultaneous differentiation of multiple tissue types from single PSC-derived EBs has been achieved through appropriate signaling regulation. For instance, Silva et al.[210] reported generation of multilineage organoids containing both cardiac and gut tissues using single mesendoderm progenitor spheroids. Their finding demonstrated that gut tissues play a role in supporting the structural and functional maturation of cardiac tissues. Likewise, human cardio-pulmonary organoids were created, revealing that the cardiac lineage contributes to alveolar maturation.[211] These interactions with other tissues can enhance the maturity of specific organoid types. Considering the impact of pulmonary diseases on other

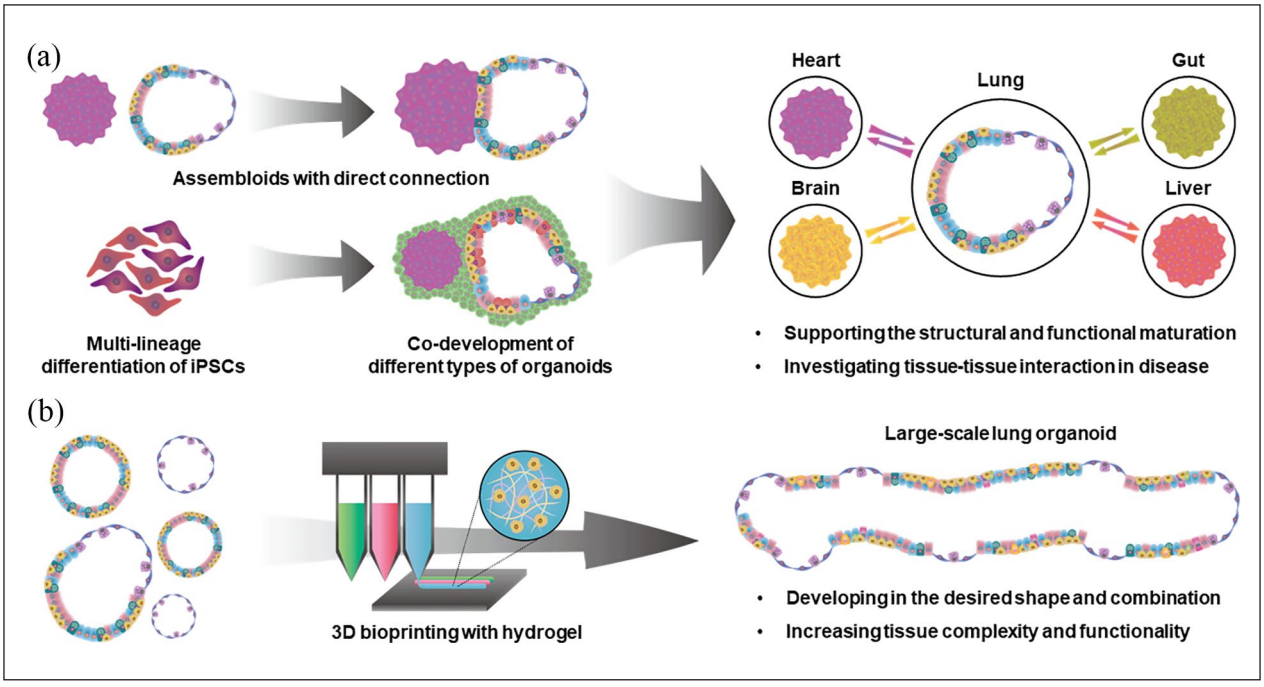


**Figure 6.** Engineering strategies using advancing organoid platforms: (a) direct organoid assembly and simultaneous development of different types of organoids to study interactions between different tissues and boost the structural and functional maturation of lung organoids and (b) fabrication of large-scale lung organoid structures with enhanced complexity and functionality using 3D bioprinting in conjunction with hydrogel.

organs beyond the lungs, particularly the heart and brain, due to abnormal gas exchange and dysfunction,[212–214] assembloids and multi-tissue models stand as powerful tools. These models possess immense potential for exploring the systemic effects triggered by pulmonary diseases and unraveling their intricate mechanisms. Thirdly, the integration of new bioengineering techniques is necessary to further enhance the environmental, structural, and physiological resemblance between lung organoids and native lung tissues. To date, ALI approach has been employed to expose cells to air in a simple 2D monolayer state in transwell or chip. However, the implementation of ALI in 3D lung organoids remains a challenge. In particular, extending the ALI technique to the apical side of 3D lung organoids presents a substantial challenge, primarily due to the enclosed nature and small size of the lumen within lung organoids.[178] This limitation may be overcome through the application of 3D bioprinting, which allows for the creation of the desired large-scale 3D structures (Figure 6(b)). A 3D bioprinting technology has already been used to produce sizable intestinal, kidney, and lung structures, enabling co-culture with their niche cells (e.g. intestinal mesenchymal cells and lung fibroblasts).[154,215–218] Typically, 3D bioprinting involves the extrusion of bioink containing cells embedded within various hydrogels such as alginate, fibrin, and gelatin methacryloyl.[219,220] Although current bioprinting technique is still insufficient to replicate the intricate structure of native lung tissue, ongoing research is focused on improving the printing resolution and the hydrogel properties.[221] Additionally, incorporation of repetitive mechanical tension into lung organoids is required as this stimulus is known to be important for lung regeneration and disease progression.[222,223] Addressing these aspects is expected to further improve the physiological relevance of lung organoids to human lung tissues.

Finally, several challenges should be addressed to facilitate the practical application of the lung organoid-based disease models. To increase the full potential of the lung organoids to accurately predict drug responses as a preclinical model prior to human clinical trials, the reproducibility and standardization of lung organoid-based disease models should be guaranteed. In this perspective, the development of automated organoid culture and analysis platforms that enable scalable, reproducible organoid production and high-throughput drug testing will augment the practical utility of lung organoid models in drug discovery and expedite their commercialization.[224] Additionally, the clinical relevance of the organoid disease models needs to be carefully verified via direct comparison studies with the patients' phenotypes and symptoms which can demonstrate correlations between clinical data and lung organoid-derived outcomes. In tandem with understanding the pathological factors of the disease, it is pivotal to reflect the progression and trajectory of the illness.[225] Chronic lung diseases, such as IPF and COPD, progress with symptoms persisting over several years.[198] Unlike infectious

diseases that occur abruptly, these conditions arise from continual and prolonged exposure to causative factors.[226,227] Consequently, modeling chronic lung diseases with lung organoids may necessitate long-term maintenance and observation, which could be realized via long-term exposure to chronic factors and integration of culture platforms like organoid-on-a-chip. This spatiotemporal modeling would better recapitulate the symptoms and physiological features of lung diseases in reality.

## Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the National Research Foundation of Korea (NRF) grants (2021R1A2C3004262 and 2022M3A9B6082675) funded by the Korea government, the Ministry of Science and ICT (MSIT). This research was also supported by the Yonsei Signature Research Cluster Program of 2023-22-0012 and the Yonsei Fellow Program funded by Lee Youn Jae.

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