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REVIEW

Design and realization of lung organoid cultures for COVID-19 applications

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Abstract
Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2, has spread globally and threatens public health. Advanced in vitro models that recapitulate the architecture and functioning of specific tissues and organs are in high demand for COVID-19-related pathology studies and drug screening. Since three-dimensional in vitro cultures, such as self-assembled and engineered organoid cultures, surpass conventional two-dimensional cultures and animal models with respect to increased cellular complexity, an environment more relevant to humans, and reduced cost, they are promising platforms for understanding viral pathogenesis and developing new therapeutics. This review highlights the recent advances in self-assembled and engineered organoid technologies that are used for COVID-19 studies. The challenges and future perspectives are also discussed.

Keywords Lung organoid · COVID-19 · Self-assembled organoid · Engineered organoid

Introduction

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and has spread rapidly worldwide, affecting millions of people. The dominant site of infection for SARS-CoV-2 is the human lung, a major component of the respiratory system responsible for mediating gas exchange into the bloodstream via alveoli. Following the invasion of the respiratory system, the virus attacks pulmonary cells, particularly alveolar epithelial type 2 (AT2) cells, by binding its viral spike protein, which is activated via proteolytic cleavage by type 2 transmembrane serine protease (TMPRSS2), to angiotensin-converting enzyme 2 (ACE2) receptors on the host cell membrane (Fig. 1a) [1]. This results in a variety of symptoms, such as cough and fever (mild), respiratory failure, hypoxemia, and pneumonia (severe), and even septic shock and organ failure (critical) [2].

Given the severity of the COVID-19 pandemic, research models and platforms, from simple to advanced, are essential for studying viral pathogenesis, prophylactics, and therapeutics. Two-dimensional (2D) cell culture systems are most commonly used, in which cells are cultured as a monolayer in flat culture flasks or Petri dishes [3]. The simplicity and relatively modest cost of 2D cell cultures make them an attractive option [4]. Due to the uniformity of 2D lung cultures, they have been employed for studying cellular differentiation and tissue response and for disease modeling [4, 5]. Two-dimensional culture models have been used to study COVID-19 pathogenesis, including SARS-CoV-2 biology, replication, and host cellular responses [6, 7]. However, they fail to fully recapitulate the physiologically relevant dynamics and complexity of SARS-CoV-2 infection. For example,

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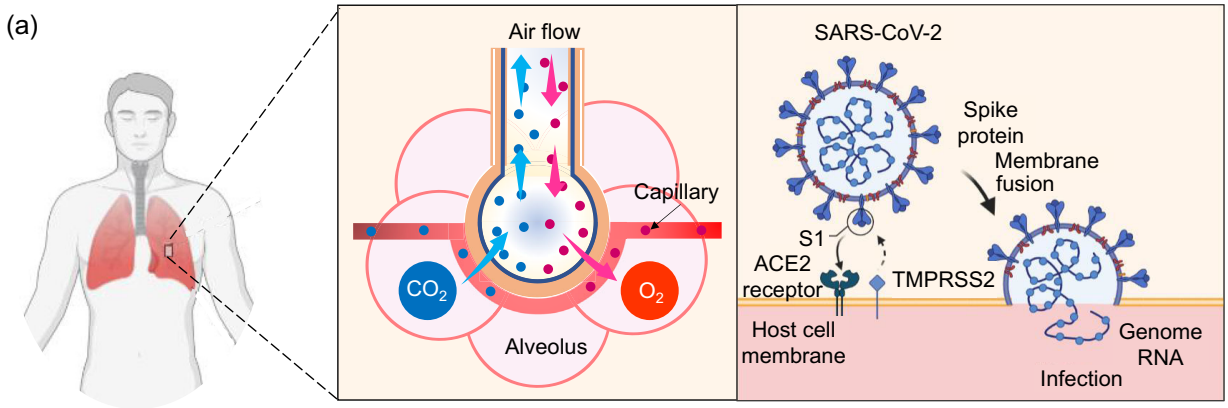

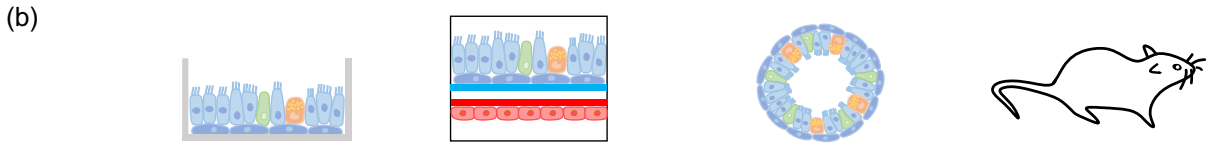

	2D model	3D engineered ALI model	3D self-assembled organoid model	Animal model
Pros	High reproducibility	Physiological relevance with air and liquid interface	High physiological relevance	High complexity of in vivo environment
	Capability for high-throughput experiments	Multiple cell co-culture	Increased complexity and heterogeneity	Systematic modeling of a whole organism with vessels and immune system
	Methodological simplicity and low cost	Controllable environment	More accurate modeling of developmental biology	Capability for long-term experiments
Cons	Lack of cellular microenvironment	Lack of vessels and/or immune system	Lack of vessels and/or immune system	Differences in predicting human responses, safety, and efficacy
	Limited physiological relevance and complexity	Limited physiological complexity	Low reproducibility and methodological complexity	Limited high-throughput experiments
	Limited cell-to-cell interactions	Limited cell-to-ECM interactions	High cost and slow culture formation	High cost and time consuming

Fig. 1 **a** Illustration of the human alveolar structure and the SARS-CoV-2 infection process. **b** Summarized advantages and disadvantages of different models. SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; ACE2: angiotensin-converting enzyme 2; TMPRSS2: type 2 transmembrane serine protease; ALI: air–liquid interface; ECM: extracellular matrix

cellular communications and outputs are not accurately represented in high-throughput screening. Thus, experiments with 2D cultures may lack translational and clinical correlations.

Animal models provide an alternative to 2D cultures and yield unique insight into complex biological systems, thereby elucidating viral pathogenesis and providing a means of evaluating pharmacologic responses [8]. Fortunately, since the entire genome of the COVID-19 virus is >80% similar to that of SARS-like bat CoV, previous animal models used for SARS-CoV studies can be utilized to study the infectivity and pathogenicity of SARS-CoV-2 [9]. Animal models of human disease are expected to have similar routes of infection, disease severity, and morbidity and mortality levels to those observed in humans [10]. Rhesus macaques [11], transgenic mice [12], and ferrets [13] were among the first animal models used for studies on SARS-CoV-2 infection, as well as for drug screening and vaccine testing. Thus far, animals that are susceptible to or have been used as models to study SARS-CoV-2 include cats, ferrets, fruit bats, human ACE2

(hACE2)-expressing mice, hamsters, tree shrews, and non-human primates [10]. The advantages and disadvantages of various animal models have been well summarized, and these models have been further refined to explore several important aspects of COVID-19, such as its pathology, transmission, and host responses to SARS-CoV-2, as well as the safety and efficacy of potential therapeutics and vaccines [14–17]. However, the genetic differences between humans and animals mean that animal models are incapable of fully mimicking human physiological responses. To date, there is no perfect animal model that completely replicates the human response to SARS-CoV-2 due to the anatomical differences between animal and human respiratory systems [14, 18].

The limitations of 2D and in vivo animal systems have motivated researchers to develop and improve three-dimensional (3D) in vitro culture systems. The use of an extracellular matrix (ECM) or scaffolding guides the formation of multiple cell types to create a 3D architecture. Generally, 3D culture systems include various features of biological systems, such as cell-to-cell and cell-to-ECM interactions, tissue-specific stiffness, oxygen and nutrient gradients, and the cooperation of tissue-specific multiple cells, thereby representing the in vivo cellular microenvironment [19, 20]. Since the cells are properly oriented, 3D models are more physiologically accurate for clinical applications. Extensive research has been performed, and biomaterials, such as collagen and Matrigel, have been utilized as scaffolds to successfully generate lung organoids [21]. Three-dimensional models can aid in elucidating the mechanism of SARS-CoV-2 infection, and they promote therapeutics research. In particular, cellular self-assembled organoids and engineered organoids have demonstrated substantial benefits in viral disease studies and appear to be promising approaches for COVID-19 studies. For simplicity, such organoids are collectively referred to as lung organoid cultures. The advantages and disadvantages of various models are summarized in Fig. 1b.

While typical 3D cell culture models [22, 23] and various types of human stem cell-derived organoid models [24] have been reviewed for COVID-19-related applications, our report is unique due to its classification of lung organoid cultures (cellular self-assembled organoids vs. engineered organoids) and explanations of state-of-the-art techniques used for organoid culture design, cell sources, and ECM. This information may help researchers across diverse disciplines communicate more effectively and create more advanced 3D organoid cultures for studying lung diseases in general. Furthermore, the challenges and future perspectives are discussed in terms of physiologically relevant complexity and functionality, advanced ECM, organoids-on-a-chip, viral infection effectiveness, and high-throughput drug screening.

3D lung organoid cultures for COVID-19-related applications

Background of lung organoid cultures

The increasing need to screen and develop drugs to treat and prevent COVID-19 has led to the widespread use of organoid cultures as fast, efficient, and accurate systems to study the biology of SARS-CoV-2, cellular tropism, and candidate drug efficiency [25]. Therefore, refining 3D organoid culture systems is currently a major area of exploration. The "oid" in the term "organoid" originates from the Latin word "*oides*," meaning "to resemble." Generally, a traditional organoid is defined as a 3D self-assembling organ-like miniature structure that is usually cultured from stem or progenitor cells into an in vitro multicellular model with organ-specific cell types, structures, and functions. While having the same application objective, engineering strategies, such as the organ-on-a-chip approach, create multicellular 3D in vitro models directly without relying on cellular self-assembly. Herein, we have classified organoid cultures as cellular self-assembled organoids or engineered organoids (Fig. 2). The term "self-assembled organoid" refers to the traditional organoid to distinguish it from other engineering efforts to make organoid cultures/models or mimics for COVID-19-related applications.

Self-assembled organoid cultures are 3D in vitro miniature organs that are formed by multicellular self-organization, which derives from the characteristic proliferation and/or differentiation of human cells (mostly human stem cells) [26, 27]. Since it is complex and challenging to fully mimic the biochemical environment of in vivo systems, organoid cultures are simplified models that accurately recapitulate the architecture and physiology of specific organs [28]. The first organoids were developed in 2009 [29]. Since then, organoids have been cultured to mimic a multitude of organs, including the lung, and the future looks promising for the successful culture of any organ in vitro [30].

In addition to self-assembled organoids, engineered in vitro organoid models, such as Transwell air–liquid interface (ALI) models, microfluidic or organ-on-a-chip models, and 3D-printed models, have been developed for lung-related studies [31–34]. In the past few years, elaborately designed lung-on-a-chip models have been reported to successfully reconstitute the periodic breathing activity of the living lung and its microstructure, as well as the dynamic microenvironment of the alveolar–capillary unit [35–38]. In response to the serious global COVID-19 pandemic, microfluidic lung chips were created to model organ-level lung infection and immune responses [39–42]. Compared with animal models, an advantage of such in vitro models is that a specific factor can be separated from the complex in vivo environment to clarify its single contribution.

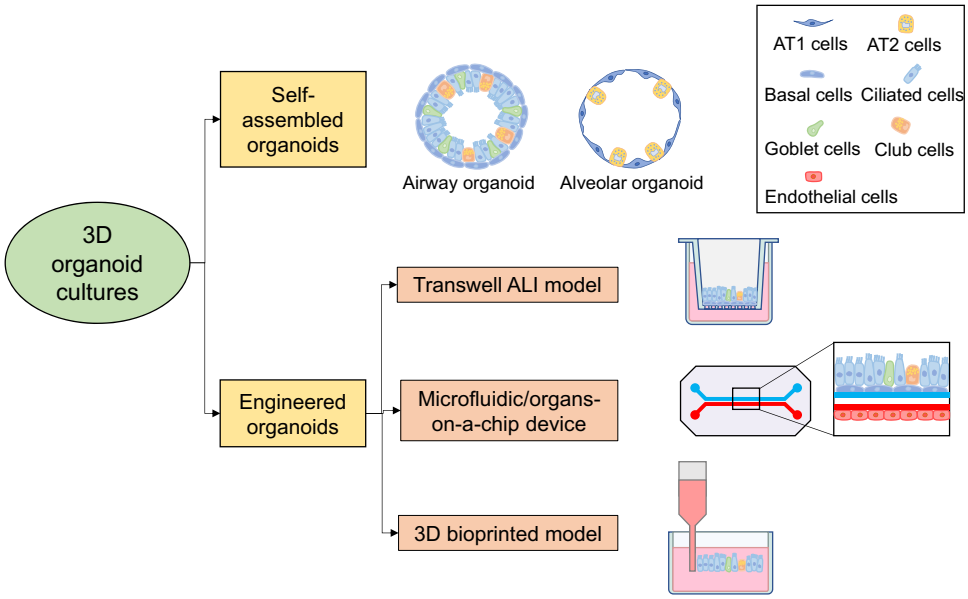

Fig. 2 Classification of typical 3D lung organoid cultures. ALI: air–liquid interface; AT1: alveolar epithelial type 1; AT2: alveolar epithelial type 2

Currently, cellular self-assembled and engineered organoid culture models are deemed the most promising models for COVID-19 and future similar applications, yielding valuable information about kinetics, pathogenesis, and host responses. The creation of models that mimic the in vivo environment as accurately as possible is valuable because these would surpass the utility of traditional 2D cell cultures and animal models.

Requirements of a physiologically relevant COVID-19 lung organoid culture

Lung organoids have various designs depending on the region of the lung to be modeled and can include both proximal and distal components. The starting cell type and culture microenvironment used to support proliferation and differentiation determine the eventual application of a lung organoid [43]. Certain physiological, cellular, and molecular features are required to successfully recapitulate the physiological environment and simulate the functions of the human lung for COVID-19 research applications.

Descending into the trachea, which branches into the left and right bronchi, and down to the alveoli, which are the smallest components of the respiratory tract, the cellular compositions of these lung areas are distinct. The airways comprise basal, club, ciliated, and goblet epithelial cells, while the alveoli contain alveolar epithelial type 1 (AT1) and AT2 cells. AT1 cells provide the surface area for the sacs to expand, while AT2 cells secrete surfactants and proteins to lower surface tension and prevent alveolar collapse. Thus, the type of lung organoids created depends on the location targeted for modeling. Each lung organoid is a model of either a section of the conducting zone or the respiratory zone. The conducting zone includes all structures that form a passageway for the transport of air, while the respiratory zone includes all structures that participate in gas exchange. Epithelial cells in both regions are the primary targets of SARS-CoV-19.

An ideal lung model used to study SARS-CoV-2 pathogenesis and evaluate candidate therapeutics is expected to physiologically mimic the viral infection and replication processes. Regarding the choice of cell types, human stem cells, such as human pluripotent stem cells (hPSCs) and adult stem cells (ASCs), are promising due to their unlimited proliferative capacity, their potential to yield all cell types, and their ability to undergo genetic modification using clustered regularly interspaced short palindromic repeats/Cas9 editing [25]. Besides stem cells, immortalized human cell lines can also be used, such as BEAS-2B cells [44] or human bronchial epithelial cell lines [45] to model the airway epithelium, and immortalized human alveolar epithelial cell lines to model the alveolar epithelium [46]. Furthermore, epithelial cells can be combined with fibroblasts and/or endothelial cells to model mesenchyme and blood vessels. A potential benefit of using cell lines representing individual cell types is that each type can be independently genetically modified, facilitating studies on individual components. Since ACE2 receptors and TMPRSS2 are required for SARS-CoV-2 binding and successful replication following invasion into the host cells [47], a physiologically relevant lung organoid culture suitable for COVID-19 research must contain epithelial cells expressing ACE2 receptors as well as TMPRSS2.

Thus, ideal lung organoids must include certain epithelial cells (cellular requirements), have an ALI for gas exchange (physical requirement), and express ACE2 and TMPRSS2

(molecular requirements), which are necessary for SARS-CoV-2 infection. With these requirements in mind, organoid cultures are expected to lead to an improved understanding of the underlying mechanisms of SARS-CoV-2 infection and treatment.

Development of lung organoid cultures

Self-assembled lung organoids

To date, the most significant and widely studied lung organoids constructed for COVID-19 research include four main types: airway, alveolar, bronchial, and bronchioalveolar organoids. Additionally, the invasion of other organs by SARS-CoV-2 has been modeled using intestinal, kidney, liver, and brain organoids [48]. Per the scope of this review, only the progress of airway, alveolar, bronchial, and bronchioalveolar organoids is discussed herein. Regarding the evaluation methods, immunofluorescence and scanning electron microscopy study cytopathic effects, whole-genome sequencing and reverse transcription quantitative polymerase chain reaction determine viral replication kinetics and genetic alterations, transcriptomic profiling reveals the differential expression of genes related to viral infection, and flow cytometry detects and quantifies cells before and after SARS-CoV-2 infection [49].

Self-assembled organoid cultures

Airway and bronchial organoids Airway organoids are 3D structures comprising differentiated epithelial cells. They mimic the cellular environment and organization of the upper airways of the human lung and are a useful tool for studying human lung diseases and COVID-19 [50, 51]. The airway epithelium, mainly containing goblet, basal, club, and ciliated cells, is the first line of defense against airborne pathogens and protects the alveoli, which constitute a vulnerable surface area approximately the size of half a tennis court in an adult human [52, 53]. Other cell types, including pulmonary neuroendocrine cells and ionocytes, also exist in this region, but they are rare. Airway organoids derived from human lung tissues can morphologically and functionally simulate the human airway epithelium, enabling assessment of the infectivity of emerging influenza viruses [54]. Typically, basal cells are on the outer layer of airway organoids, while the multiciliated cells are situated on the luminal side (Figs. 3a and 3b) [50]. Since the onset of the COVID-19 pandemic, airway organoid models infected with SARS-CoV-2 have been used to study the viral replication kinetics, tropism, and host response [49]. For example, ciliated cells and club cells were found to be susceptible to SARS-CoV-2 infection in airway organoids that were generated from human embryonic stem cells (hESCs) and contained cellular constituents of basal cells, ciliated cells, club cells, and goblet cells [55]. Using this model, the antiviral performance of drugs, such as remdesivir, was evaluated after infection with SARS-CoV-2, demonstrating its potential as a drug screening platform. Moreover, it is expected that airway organoid models will be used to study the immunological responses to SARS-CoV-2 when cocultured with different immune cells [49].

Since severe acute bronchopneumonia frequently occurs in COVID-19 patients, bronchial organoids, which mimic the small airways of the respiratory tract, have been used to clarify the mechanism underlying the destruction of the bronchial epithelial layer [56, 57]. To reproduce the infection of SARS-CoV-2 in the bronchi, Sano et al. [56] used cryopreserved normal human bronchial epithelial cells (NHBEs) to generate human bronchial organoids composed of basal, ciliated, goblet, and club cells. They observed that ciliated cells infected with the virus died, while basal cells survived after viral infection and differentiated into ciliated cells, possibly under the regulation of fibroblast growth factor 10 signaling. Fang et al. [58] used infected and uninfected bronchial organoid models generated from primary human bronchial epithelial cells (hBEpCs) to investigate targeting colony-stimulating factor 3, which was identified as an upregulated gene, as a potential therapeutic approach.

Alveolar organoids Alveolar organoids aim to model the alveoli, which are the small sacs that terminate the branching of bronchioles. These organoids have been applied to elucidate the pathogenesis of human lung failure caused by SARS-CoV-2. Alveolar organoids differ dramatically in their cellular composition and function compared with airway organoids. In alveoli, cuboidal AT2 cells account for approximately half of the alveolar epithelial cells and secrete pulmonary surfactant, while flat, delicate AT1 cells cover most of the alveolar surface, mediating gas exchange to adjacent capillary vessels and serving as an important component of the blood–air barrier [59]. Alveolar organoids are composed of AT1 and AT2 cells and may also contain mesenchymal cells. Pei et al. [55] derived human alveolar organoids that contained AT1 and AT2 cells from hESCs and found that SARS-CoV-2 infected the AT2 cells due to their high expression levels of ACE2 receptors and TMPRSS2. Han et al. [60] utilized hPSCs to create an alveolar organoid model. They determined that the inflammatory changes observed in human COVID-19 infection were mimicked by the upregulation of cytokine/chemokine signaling, and treatment with identified drugs significantly inhibited SARS-CoV-2 infection. Similarly, Tiwari et al. [61] generated human induced pluripotent stem cell (iPSC)-derived lung organoids (Figs. 3c and 3d) and found that they were permissive to SARS-CoV-2 infection due to highly expressed

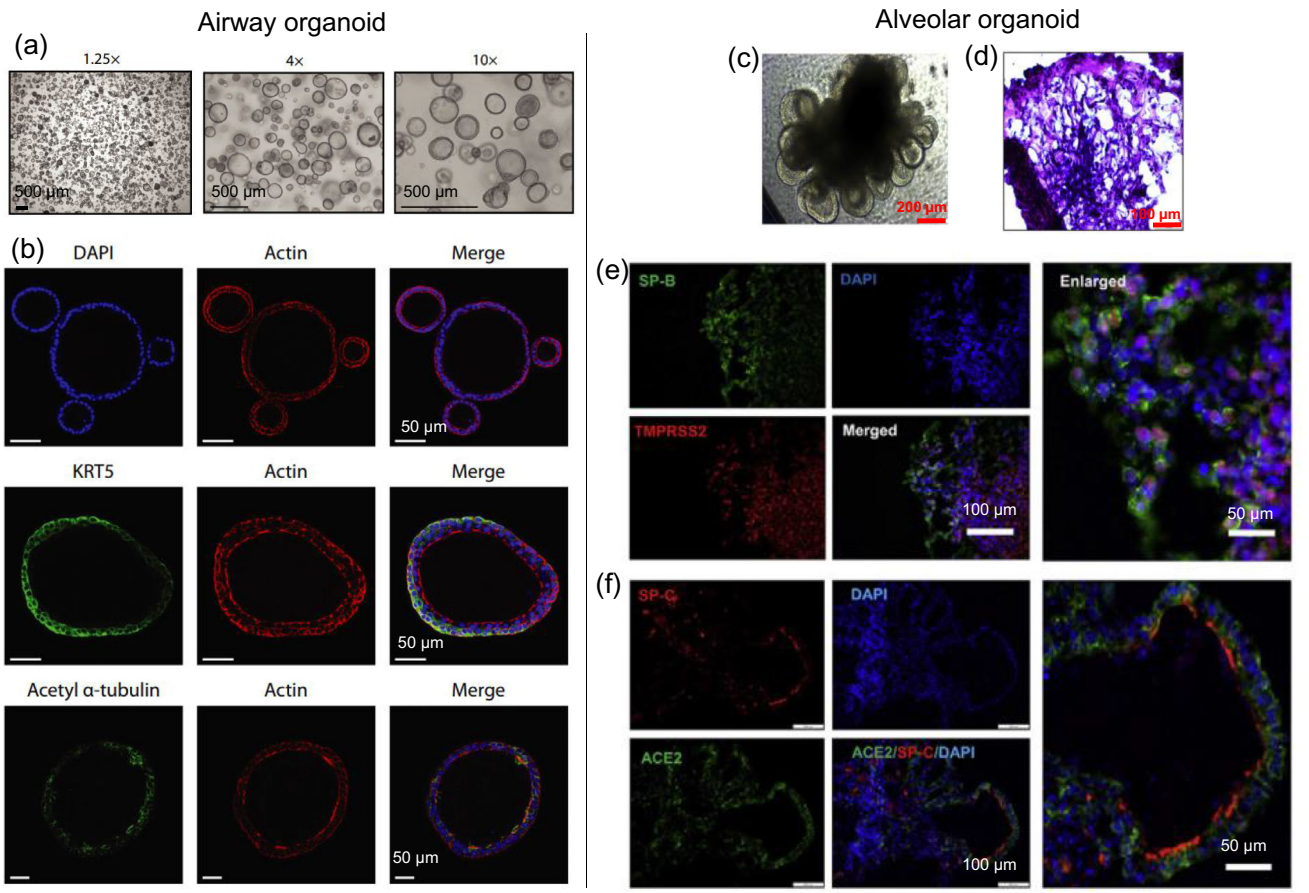

Fig. 3 Lung organoid cultures. Left column: airway organoid. Reproduced from [50], Copyright 2020, with permission from the Association for the Publication of the Journal of Internal Medicine. **a** Brightfield images of airway organoids. **b** Confocal images of the organoid showing cytokeratin 5 (KRT5, basal cell marker) and acetylated α-tubulin (ciliated cell marker). Right column: alveolar organoid. Reproduced from [61], Copyright 2021, with permission from the authors, licensed under CC BY-NC-ND 4.0. **c** Phase-contrast image of lung alveolar organoids. **d** Hematoxylin and eosin (H&E) staining of alveolar-like morphology. **e**, **f** Confocal images showing AT2 cells (surfactant protein B (**e**) and surfactant protein C (**f**)) colabeled with TMPRSS2 (**e**) and ACE2 (**f**). TMPRSS2: type 2 transmembrane serine protease; ACE2: angiotensin-converting enzyme 2; DAPI: 4',6-diamidino-2-phenylindole

TMPRSS2 (Fig. 3e) and ACE2 (Fig. 3f). Collectively, these studies have verified the fidelity of the alveolar organoid disease models to investigate the human lung pathophysiology of SARS-CoV-2 infection as well as to screen new drugs.

Bronchioalveolar organoids Although bronchioalveolar organoids are less common, they are significant because they serve as models for cellular tropism analysis and treatment testing. Bronchioalveolar organoids model the alveoli and the respiratory bronchioles, which are conjoined at the bronchioalveolar duct junction. Lamers et al. [62] developed a bronchioalveolar-like model from human small airway stem cell-derived lung bud tip progenitor organoids that comprised both alveolar-like and bronchiolar-like cells, merging the alveolar organoid and bronchiolar organoid models to resemble the bronchoalveolar duct junction. The cells present in this region included AT1 and AT2 cells, club cells, ciliated cells, basal cells, and neuroendocrine cells. SARS-CoV-2 was accurately replicated within these organoids, proving that the model is ideal for virus infection and drug screening.

Typical cell sources and ECM

Various cells and ECMs have been utilized for the development of lung organoid cultures (Table 1).

Cell sources Lung organoids are extremely useful models for COVID-19 research, providing a greater understanding of pathogenesis and effective therapies. The replication of both the histology and function of the lungs enables the in-depth examination of COVID-19 kinetics, tropism, and host responses [34]. Current lung organoid cultures are usually derived from hPSCs or ASCs with inductive factors and a supportive 3D environment. hPSCs, including iPSCs

Table 1 Typical cell sources and ECMs used for establishing human lung organoid cultures

Human lung organoid	Cell source	ECM	Reference
Airway and bronchial organoid	Solid human lung tissue	Matrigel	[54, 63]
	Human lung fibroblasts	Matrigel	[64]
	Human lung microvascular endothelial cells		
	NHBEs		
	NHBEs	Matrigel	[56]
	hBEpCs	NA	[58]
Alveolar organoid	hPSCs	Matrigel	[24, 65]
	iPSCs	Matrigel	[61]
	Immortalized human alveolar epithelial cell lines	Matrigel	[46]
Bronchioalveolar organoid	Human small airway stem cells	Matrigel, collagen I	[62]

ECM: extracellular matrix; NHBEs: normal human bronchial epithelial cells; hBEpCs: primary human bronchial epithelial cells; NA: not applicable; hPSCs: human pluripotent stem cells; iPSCs: induced pluripotent stem cells

and hESCs, are the most common starting cell type used to encourage proliferation and differentiation into a lung organoid [43]. Due to the limited availability of primary human lung samples, hPSCs are the most promising because they can differentiate into cell types that are found in the organ environment [66]. The regeneration of lung models requires the differentiation of multiple cell types to replicate the cellular architecture and function. Chen et al. [67] generated lung bud organoids using hPSCs that developed into branching airways and early alveolar structures over a 20-d period. Since the emergence of the COVID-19 pandemic, iPSCs and hESCs have been used to derive human lung organoids for COVID-19 infection and drug testing studies [55, 61].

Cells from adult tissues can also be used to prepare organoids. For example, Tan et al. [64] incorporated NHBEs, lung fibroblasts, and lung microvascular endothelial cells into a 3D multicellular environment to generate airway organoids and found that the randomly mixed cell populations self-organized into discrete epithelial and endothelial structures. Sachs et al. [63] reported a versatile approach to establishing adult human airway epithelial organoids containing basal cells, functional multiciliated cells, mucus-producing secretory cells, and CC10-secreting club cells from patient materials (such as lavages, biopsies, and resections). Human lung epithelial cells, such as NHBEs [56] and hBEpCs [58], have successfully been used to study COVID-19.

Extracellular matrix The ECM is a 3D network that provides a scaffold for cellular attachment and supports signaling among different cell types. The complex function of native ECM means that a model capable of fostering long-term expansion of basal cells while maintaining their phenotypic stability has not yet been developed. Since ECM composition changes throughout the phases of lung development, ranging from fetal to neonatal and, finally, adult tissue, the replication of ECM is challenging [68]. Natural matrices in which cells can be successfully embedded, such as Matrigel, fibrin, and collagen, have been explored as culture environments and altered to mimic characteristics of native ECM. Among the different matrices, Matrigel is currently the most widely used ECM material for organoid cultures [67, 69]. However, further studies are necessary to obtain a more accurate understanding of how a specific ECM affects and facilitates cellular differentiation.

Engineered lung organoids

Transwell ALI models

When COVID-19 emerged, self-assembled organoids were widely cultured in a submerged Transwell ALI setting, which encourages the proliferation and differentiation of stem cells, followed by the formation of a biomimetic ALI structure [6, 7, 70–72]. In particular, 3D ALI models enable cellular interactions on a semi-dry cell surface, consisting of an apical surface exposed to air and a basal surface submerged in a liquid culture medium (Fig. 4a) [73]. As opposed to traditional fully submerged cell cultures, ALI cultures directly mimic the in vivo structure of the respiratory tract [74, 75]. The ALI cultures comprise cells that are more irregular in shape with rougher surfaces and an abundance of secretions, which are superior to the smooth and less matured cells in submerged cultures [76]. Additionally, since multicellular ALI models are composed of more than one cell type, they better resemble the cellular arrangement in vivo compared with a monoculture. Thus, they have been used to study disorders of the respiratory tract in response to viral infection [31].

Overall, both 2D and 3D ALI models are used to study the cell biology of the respiratory system and model respiratory diseases and infections, as well as for drug discovery. They have also been used for COVID-19 studies. Sano et al. [56] compared the SARS-CoV-2 infection efficiency between the spherical bronchial organoid from NHBEs and the suspended

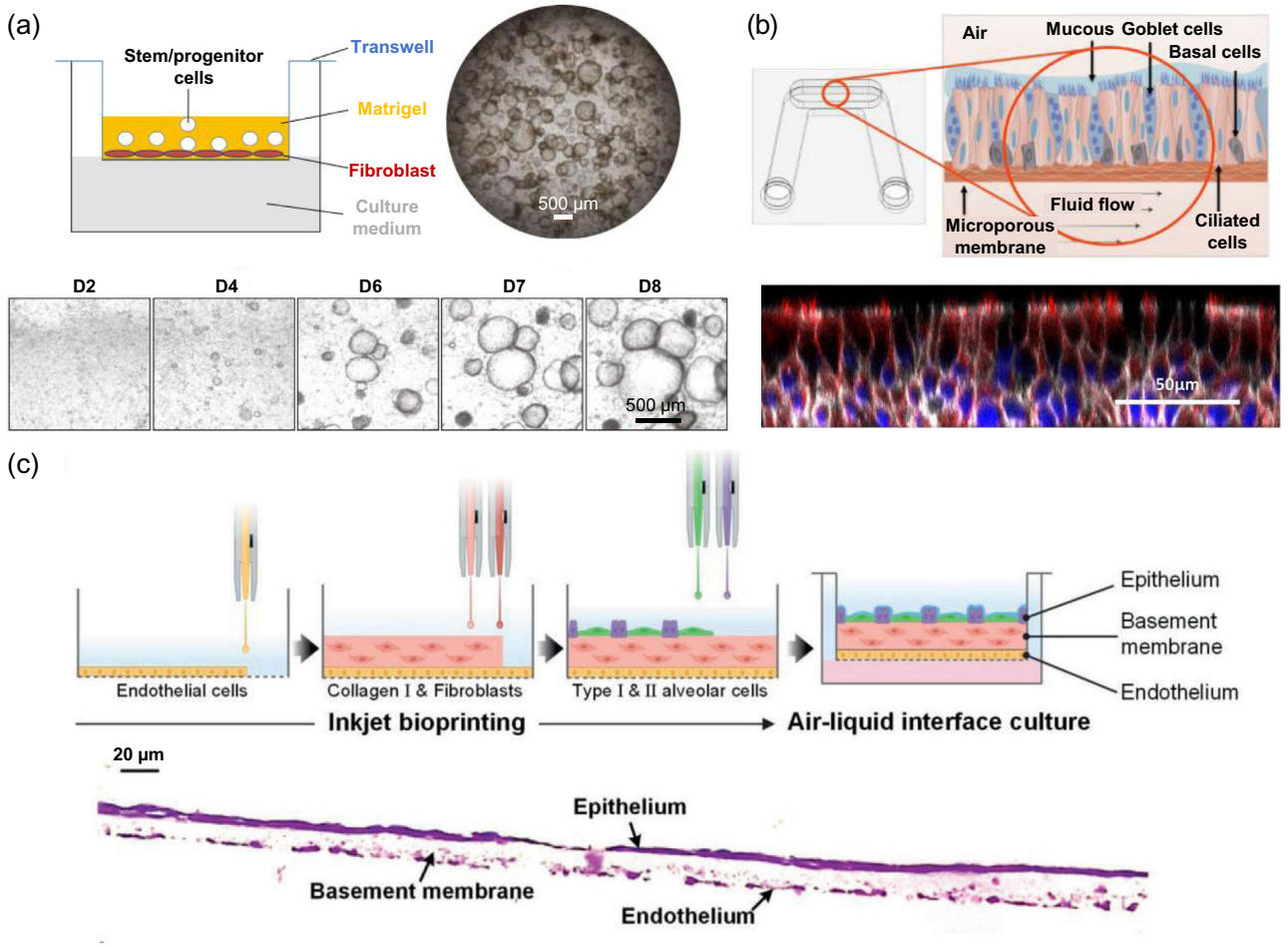

Fig. 4 Approaches for lung organoid engineering. **a** (upper) Schematic of an ALI organoid culture loaded into a Transwell filter insert and (lower) images of the organoids over an 8-d period after seeding. Reproduced from [73], Copyright 2020, with permission from the authors, licensed under CC BY 4.0. **b** (upper) Schematic of an ALI microfluidic platform including differentiated cell populations and basolateral fluid flow and (lower) a confocal image of the pseudostratified epithelium established on an ALI. Reproduced from [75], Copyright 2021, with permission from the authors, licensed under CC BY 4.0. **c** (upper) Schematic of the inkjet printing process for fabrication of a 3D alveolar barrier model and (lower) an H&E-stained cross-sectional image of the alveolar barrier model showing the epithelium, basement membrane, and endothelium layers. Reproduced from [88], Copyright 2021, with permission from the authors, licensed under CC BY 4.0. ALI: air–liquid interface; H&E: Hematoxylin and eosin

organoid in ALI culture. They found that the ALI model was considerably more efficiently infected due to viral infection from the luminal side. Intriguingly, Sasaki et al. [77] reported that A549 alveolar epithelial cells in ALI culture, as opposed to those in conventional submerged cultures, became susceptible to SARS-CoV-2 infection due to the increased expression of ACE2 and TMPRSS2.

Lung-on-a-chip models

The development of microfluidic or organ-on-a-chip devices provides better organ-level functionalities required to serve as a disease model and maintain differentiation and tissue-specific functions. Organ-on-a-chip devices are usually microfluidic-based, allowing continuous perfusion of medium and/or air to recirculate nutrients, remove waste products, and oxygenate the media. Since lung-on-a-chip integrates multiple physiological components of an in vitro system, such as the ALI structure and flow, their replication of the microenvironment is physiologically relevant. Huh et al. [36] developed a multifunctional microdevice that reproduces key structural, functional, and biomechanical properties of the human lung alveolar–capillary interface, which is the fundamental functional unit of a living lung. Their micro-engineered lung-on-a-chip contained channels that were lined with mammalian lung epithelial and endothelial cells exposed to air, fluid flow, and cyclic mechanical

strain, mimicking normal breathing motions. Uniquely, this microdevice recreated the physiological breathing movements observed in the human lung while providing oxygenated air to the cells as well as causing physical stretching of the membrane. Results demonstrated that cyclic mechanical strain accentuated toxic and inflammatory responses of the lung to silica nanoparticles, as well as epithelial and endothelial uptake of nanoparticles. Moreover, it reproduced complex integrated organ-level responses to bacteria and inflammatory cytokines introduced into the alveolar space, leading to the discovery of new therapeutic agents.

Lung-on-a-chip models have had extraordinary implications in COVID-19 research regarding pathogenesis and the development of effective therapies [34, 78]. Generally, the microfluidic aspect fosters a multicellular culture in an environment that more accurately resembles in vivo functions and interactions with surrounding systems. These advantages enable the analysis of SARS-CoV-2 and its pathological interactions with existing chronic respiratory diseases in a host. Si et al. [41] developed an airway chip using primary human lung bronchial-airway basal stem cells cultured on one side of the membrane (named the "airway channel") and a primary human lung endothelium cultured on the other side (named the "vascular channel"), which was exposed to a continuous medium flow. They found that the antiviral performance of drugs tested on the chips infected with pseudo-typed SARS-CoV-2 appeared more clinically relevant than when cells were cultured under static conditions. Gard et al. [75] fabricated a microfluidic ALI culture platform by culturing NHBEs (Fig. 4b) to evaluate viral infection kinetics and antiviral agent dosing. They demonstrated that SARS-CoV-2 infected the airway epithelial cells of the model via ACE2 receptors and TMPRSS2.

3D-printed models

3D bioprinting is one of the most promising technologies for tissue engineering [79, 80]. This process prints biological materials and living cells layer-by-layer to create biomedical models. The cells are typically suspended in a hydrogel solution at a required number and density to form bioinks for 3D printing, and certain rheology properties of bioinks are required to enable the printing and gelation [81–84]. This method of mechanical assembly creates a precise and consistent spatial pattern within a 3D culture environment. Thus, organoid bioprinting significantly eliminates variation across culture batches, which is an issue when constructing organoid cultures using conventional production methods [85]. Furthermore, bioprinting bridges the gap between the importance of cellular self-organization within cultures and the challenge of a culture self-assembling into a large and controlled tissue unit. Bioprinting techniques facilitate the natural self-organization of cells and encourage faster and more complex organoid formation [86].

Brassard et al. [87] used organoids as building blocks to 3D print certain patterns within a highly permissive ECM, demonstrating the successful integration of bioprinting and organoid technology. The pattern geometry and cellular composition were easily controlled by the extrusion printing technique. Following culture, the printed organoids showed self-organized features such as lumens, branched vasculature, and tubular intestinal epithelia, indicating the ability of printed patterns to facilitate effective multicellular self-organization and tissue morphogenesis. Kang et al. [88] used a high-resolution inkjet printing technique to fabricate a three-layered 3D alveolar barrier model by the sequential deposition of four types of alveolar cells: AT1 and AT2 epithelial cells, fibroblasts, and lung microvascular endothelial cells (Fig. 4c). Evaluation of the functional and structural characteristics of this printed 3D model revealed that the multilayered structure of the alveolar barrier showed better lung tissue morphologies and functions than conventional 2D culture models or 3D unstructured models. Thus, the production of lung organoid cultures utilizing 3D bioprinting provides an alternative tool for COVID-19 infection studies and drug discovery applications [33].

Lung organoid cultures for COVID-19 applications

Viral infection applications

Early in the COVID-19 pandemic, researchers successfully isolated SARS-CoV-2 and investigated its transmission, morphogenesis, and pathogenesis using standard immortalized cell lines, such as Vero E6 and Huh7 cells [70, 71]. However, these cells do not express ACE2 or TMPRSS2, which are the main receptors of SARS-CoV-2. Thus, Zhu et al. [7] used human airway epithelial cells to develop an in vitro infection model. Do et al. [89] cultured human tracheal airway epithelial cells and human small airway epithelial cells in an ALI-modeled system where the apical side was infected with SARS-CoV-2. Human lung organoids, as well as other organoid types with highly expressed ACE2 and TMPRSS2 proteins, have been used for SARS-CoV-2 infection studies and characterization of infection efficiencies [24, 49, 61, 90–92]. Expression of the SARS-CoV-2 nucleocapsid protein has been observed in the infected basal organoid (Fig. 5a) and AT2 organoid (Fig. 5b) after 96 h [93]. However, it must be noted that even though these epithelial cells are permissive to many respiratory viral infections, the variety of cells used in the models remains limited, and assembling all relevant cell types of the respiratory tract (such as ciliated, goblet, and basal cells) into a model remains challenging.

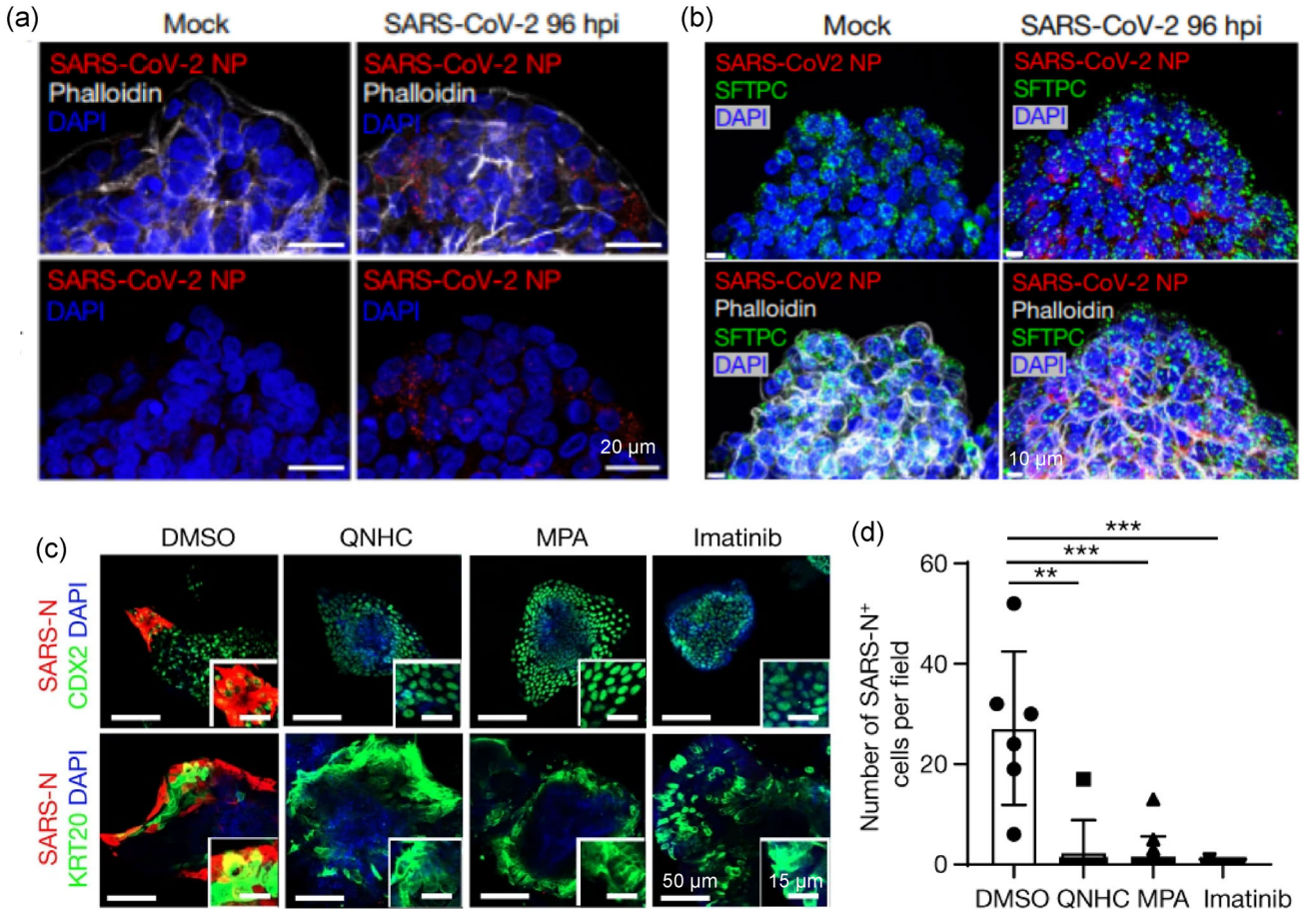

Fig. 5 Lung organoids used for infection and drug discovery applications. Lung organoids with SARS-CoV-2 infection (**a**, **b**) and drug treatment (**c**, **d**). **a** SARS-CoV-2 nucleocapsid protein appeared in basal organoids and **b** apical-out AT2 organoids at 96 h post-infection. Reproduced from [93], Copyright 2020, with permission from the authors, under exclusive licence to Springer Nature Limited. **c**, **d** QNHC, MPA, and imatinib inhibit SARS-CoV-2 infection. Data are represented as mean±standard deviation (n=6). **: P<0.01; ***: P<0.001. Reproduced from [65], Copyright 2020, with permission from the authors, under exclusive licence to Springer Nature Limited. SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; DMSO: dimethylsulfoxide; QNHC: quinacrine dihydrochloride; MPA: mycophenolic acid; DAPI: 4',6-diamidino-2-phenylindole

Drug discovery and screening applications

Lung organoid cultures infected with SARS-CoV-2 can help to identify potential therapeutic drugs against COVID-19 [60, 94]. After infection with SARS-CoV-2 or SARS-CoV-2 pseudovirus, drug candidates are applied to the infected organoids to evaluate their efficacy. One strategy to inhibit viral entry into host cells is to interfere with the binding of virus spike protein and host cell receptors. Tiwari et al. [61] reported that ACE2 and TMPRSS2 inhibitors blocked viral entry into lung organoids. Spitalieri et al. [95] used two different approaches: a neutralizing antibody and a synthetic peptide that can bind directly to the spike protein. The results showed a substantial decrease in infection efficacy in the treated cells. Han et al. [65] used a lung organoid culture derived from hPSCs as a high-throughput screening platform to identify entry inhibitors of SARS-CoV-2. They reported that treatment with identified drugs, including quinacrine dihydrochloride (QNHC), mycophenolic acid (MPA), and imatinib, significantly inhibited SARS-CoV-2 infection (Figs. 5c and 5d). Using the same lung organoid culture [65], Duarte et al. [94] screened thousands of Food and Drug Administration (FDA)-approved drugs and found that atorvastatin blocked viral entry into the lung organoid. In their search for therapeutic options, researchers have proposed the antiviral plant compound resveratrol [96–98] as an advantageous antiviral therapy for SARS-CoV-2 infection. Ter Ellen et al. [96] provided evidence that resveratrol and pterostilbene exhibited a strong antiviral effect for ALI-cultured human primary bronchial epithelial cells up to 48 h post-infection.

Challenges and future perspectives

Physiologically relevant complexity and functionality

While mimicking the whole human lung and communications between different sections of an organoid is challenging, the improved physiological relevance regarding phenotypes and functionalities of organoid cultures benefits the model's validity. Engineering a specialized lung hydrogel scaffold to guide the cells into appropriate complex branching structures merits further exploration [99]. The lack of a physiologically relevant representation of the typical lung microenvironment poses a technical drawback that may alter the host response observed in vivo following COVID-19 infection. Appropriate modeling of the roles that the immune system plays in the lungs can certainly enhance the understanding of critical events that occur after viral infection, and this may be achieved by coculturing lung organoids with immune cells and stromal cells, which reside in the peribronchovascular interstitium and interact with alveoli in vivo. Another crucial physiological process in vivo that has been largely overlooked in organoid developments thus far is vascularization [100–102]. Thus, establishing a vascularized lung organoid culture to facilitate ALI morphogenesis and cellular interactions would pave the way for further applications. A further key challenge is the inadequate standardization of protocols, which may result in abnormal self-assembled architectures with poor reproducibility.

Advanced ECMs

Optimized ECMs, allowing dynamic changes in biophysical, chemical, and mechanical properties, are necessary to recapitulate the in vivo environment. Matrigel is the most commonly utilized ECM to develop organoids, but it is isotropic and chemically undefined [103]. Thus, it may be unable to adequately simulate dynamic environmental changes with respect to the time and stage of cellular development. Additionally, xenobiotic factors within Matrigel may have unanticipated effects on the cellular responses and chemical screening results, which may pose a concern when translating the basic research findings to clinical applications. Thus, effective substitutes for Matrigel are needed.

Organoids-on-a-chip

Despite the current progress, advanced microfluidic organoid cultures for enhanced organoid maturation and branching morphogenesis require further establishment. Integrating a system where the fluidic networks replicate the natural flow conditions of blood in the surrounding capillaries, as well as including a pliable membrane to imitate the fluctuating surface area of alveoli, would ensure that mechanical forces are appropriately generated in vitro. Additionally, incorporating more flow channels to resemble different components of the lung may be beneficial in mimicking the in vivo structure. Investigation of the integration of microfluidics with self-assembled organoids to enable the fabrication of organoids-on-a-chip appears highly promising [104].

Viral infection effectiveness

Although organoid cultures serve as promising models to mimic lung functions and investigate the mechanism of infection, many challenges must be overcome before they are perfected. Variations among laboratories and protocols may lead to inconsistent infection and efficiency results. Improvements in infection efficiency in organoid models are vital to extending their scope of application. Another major challenge in the production of an effective platform is that it should not only recapitulate the pathogenesis but also allow the investigation of various aspects of toxicology, immunology, and prophylactic and therapeutic approaches. Since the complicated crosstalk between the host and the virus means that a single platform may not meet all these requirements [48], organoid culture development requires a systematic approach. Additionally, because organoids may be generated from ethnically and racially diverse patients with different health backgrounds, disparities in COVID-19 infection are expected and will need to be thoroughly understood.

High-throughput drug screening

Large-scale drug screening remains challenging for organoid technology. Although organoids have been successfully used for drug discovery [65, 105], the antiviral response and cellular tropism identified among studies using organoids were, unfortunately, inconsistent [25]. The limited batch-to-batch reproducibility of organoids hampers their application as a high-throughput drug screening approach due to variations in self-assembled cellular constitutions and architectures and intricate in vivo microenvironments. Additionally, the absence of vasculature and immune cells in current organoid cultures limits the understanding of viral infection processes and cellular responses to drugs.

Summary

The COVID-19 pandemic prompted the scientific community to explore novel technologies to fully understand the host response and potential therapies. Conventional 2D cultures and animal models have failed to adequately recapitulate the morphological and functional characteristics of in vivo human respiratory systems. Fortunately, organoids

are regarded as a promising in vitro platform for COVID-19 and similar applications because they have provided ample direction, specifically toward enhancing the understanding of viral pathogenesis and identifying candidate therapeutic agents. However, current organoid cultures still have some limitations, such as the absence of stromal tissue that includes vasculature, as well as immune and various support cells. Furthermore, more advanced models with enhanced physiological relevance, improved maturity, and better scalability are needed. Additionally, the variability of organoid cultures in terms of size, shape, and cellular composition may lead to unsatisfactory reproducibility, which is the main challenge when comparing experimental results across different application scenarios. Despite these limitations, the use of organoid cultures to study COVID-19 and other emerging pandemic diseases seems promising and highly feasible with the advances in stem cell techniques, materials science engineering, and engineering tools.

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Declarations

Conflict of interest YH is an editorial board member for *Bio-Design and Manufacturing* and was not involved in the editorial review or the decision to publish this article. The authors declare that they have no conflict of interest.

Ethical approval This study does not contain any studies with human or animal subjects performed by any of the authors.

Data availability Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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