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Studying SARS-CoV-2 Infectivity and Therapeutic Responses with Complex Organoids

Kevin G. Chen[1,*], **Kyeyoon Park**[1], **Jason R. Spence**[2]

[1]NIH Stem Cell Unit, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, USA

[2]Department of Internal Medicine, Department of Cell and Developmental Biology, and Department of Biomedical Engineering, University of Michigan Medical School, Ann Arbor, MI, USA

Abstract

Clinical management of COVID-19 patients with severe complications has been hindered by a lack of effective drugs and a failure to capture the extensive heterogeneity of the disease with conventional methods. Here, we review emerging roles of complex organoids in the study of SARS-CoV-2 infection, modelling of COVID-19 disease pathology and in drug, antibody, and vaccine development. We discuss opportunities for COVID-19 research as well as remaining challenges in the application of organoids.

INTRODUCTION

COVID-19, a SARS-CoV-2 (CoV-2) coronavirus disease, represents a global health emergency. As of May 30, 2021, approximately 169 million individuals have been infected and 3,530,582 deaths confirmed worldwide[1]. Even though vaccines have now been established to prevent infection, to date, no specific antiviral drugs exist that target CoV-2 to mitigate established disease. Clinical management of COVID-19 patients mainly focuses on improving symptoms, supporting lung function, preventing a sudden acute increase of circulating cytokines (cytokine storm), and controlling infections[2].

Ongoing fast-track clinical trials focus on therapeutic solutions that block the CoV-2 infection cycle and associated pathophysiological processes[3]. Nevertheless, it is still poorly understood how the genetic background of COVID-19 patients might affect the severity of symptoms[4,5]. Similarly, whether CoV-2-host receptor interactions might differ depending on the age, gender, and ethnicity of a patient has not been clarified. As a result, the design of effective vaccines and antiviral drugs has remained challenging. The advancement of organoid-based assays derived from human pluripotent stem cells (hPSCs) and adult stem cells (ASCs) offers an opportunity to expand and bank various types of tissue-specific

*Correspondence should be addressed to K.G.C., Kevin G. Chen, M.D., Ph.D, Address: 9000 Rockville Pike, Building 10, Room 3B14, Bethesda, MD, 20892 USA, Telephone: (301) 402-8118; Fax: (301) 480-1022, cheng@mail.nih.gov.

COMPETING INTERESTS

The authors declare no competing interests.

organoids for biomedical research[6–9]. Accordingly, stem-cell-based 2D cell cultures and 3D organoids are also used to study CoV-2 infection[10–19]. These studies highlight the need to define the roles of stem-cell-based organoids in COVID-19 research.

In this Review, we recapitulate the CoV-2 infection cycle and associated intervention strategies. We evaluate current COVID-19-based assays, focusing on their strengths and potential limitations. We further elucidate the role of respiratory cell types and lung organoids in assessing CoV-2 susceptibility and discuss other organoid systems (derived from hPSCs and ASCs) that can be used. Lastly, we examine the benefits of organoids in studying CoV-2-induced pathophysiology and predicting therapeutic outcomes.

CoV-2 infection cycle and associated intervention strategies

CoV-2 is a positive-sense and single-stranded RNA beta-coronavirus, potentially evolved from a bat coronavirus[20–23]. Genomic diversity of CoV-2 in COVID-19 patients is evident[24–26], but the environmental CoV-2 genome is relatively stable[27]. The structural genomics of CoV-2 indicates evolutionarily conserved functional regions of viral proteins[27–29]. In addition, CoV-2 shares a similar infection cycle with SARS-CoV and MERS-CoV coronaviruses[30–32].

CoV-2 infection cycle.—Just as SARS-CoV, CoV-2 enters and infects a human host cell via multiple coordinated processes[30–32]. The CoV-2 infection cycle is illustrated (Fig. 1a) with distinct steps (1–17), starting from its host cell entry via membrane fusion and endocytosis to the release of a mature CoV-2. In COVID-19 patients, the infection cycle increases the viral load in the respiratory tissues, kidneys, and intestine[33]. The induction and release of cellular cytokines (also called a cytokine storm) may trigger a wide range of host immunological and inflammatory responses in these tissues (Fig. 1b–e)[34,35]. Cytokine storms often lead to diffuse alveolar damage, acute respiratory distress syndrome (ARDS), loss of gas exchange, respiratory failure, and multi-organ damage, overall increasing death rates[35–40].

Therapeutic strategies.—Despite an insufficient understanding of the CoV-2 infection cycle, all viral processes could be conceivably employed for pharmacological, immunological, and molecular interventions of CoV-2 infections. Such experimental and clinical interventions have been reported[14,41–48], some of which are listed in Figure 1.

The abrogation of viral cell entry effectively prevents viral infection. Blockage of spike glycoprotein (S-gp) binding to ACE2 by a human recombinant soluble ACE2 (hrsACE2) reduced CoV-2 recovery from Vero cells, displaying a 1000- to 5000-fold reduction in viral growth[14]. This blockage appears to be ACE2 species-specific as recombinant mouse ACE2 had no effect[14]. TMPRSS2-mediated S-gp priming can be blocked with camostat, a clinically proven protease inhibitor, and substantially (~88%) inhibited by an anti-ACE2 antibody (at 20 μg/mL)[41]. Convalescent sera from SARS patients partially (~45%) neutralised pseudotyped CoV-2 entry[41]. CR3022, a neutralising antibody isolated from a convalescent SARS patient, targets S-gp receptor-binding domain (S-RBD) of SARS-CoV[49] and also binds to the CoV-2 S-RBD[50]. High-resolution structures revealed a mechanism by which neutralising antibodies, such as CR3022, recognise S-RBD in

its trimeric configuration[51]. Hence, these studies provide the molecular basis for future therapeutic interventions to prevent CoV-2 cell entry.

Beyond S-gp-ACE2-mediated membrane fusion, little is known about other cell entry mechanisms such as endocytic pathways, as evident in other coronaviruses. These pathways may be classified as clathrin-dependent endocytosis for SARS-CoV[52], membrane rafts and caveolar endocytosis for the human coronavirus 229E[53], and clathrin- and caveolar-independent entry of feline coronavirus[54]. Successful abolishment of CoV-2 entry by camostat suggests that the endocytic access may not be a major pathway for CoV-2 cell entry. However, this finding must be confirmed in different cellular and animal models.

The United States Food and Drug Administration (FDA)-approved drug, ivermectin, successfully inhibits the replication of CoV-2 in an *in vitro* model (i.e., Vero-hSLAM cells)[46]. The promising antiviral drug remdesivir (GS-5734), an adenosine analogue, also inhibits CoV-2 replication[44]. Remdesivir has become the first anti-CoV-2 drug approved by the FDA after a phase III clinical trial[55]. However, the World Health Organization's solidarity trial revealed that remdesivir neither reduced mortality nor shortened the recovery time of COVID-19[48]. A less toxic derivative of chloroquine, hydroxychloroquine, is an endosomal acidification inhibitor and effective in inhibiting CoV-2 infection in cell culture[42]. Hydroxychloroquine has gained wide-spread use in the treatment of COVID-19. However, its broader clinical application has been under scrutiny due to the absence of well-controlled data on its effectiveness and reported severe side effects[43].

Importantly, about 20% of severe COVID-19 cases are associated with cytokine storms, also observed in SARS and MERS[35,56], which can be treated by inhibiting cytokine release or accelerating cytokine clearance in targeted cells[57]. The monoclonal antibody tocilizumab, which inhibits IL-6, has been used to treat cytokine storms in COVID-19 patients in clinical trials[58]. In an early trial, treatment with tocilizumab reduced the risk of invasive mechanical ventilation or death rate in severe COVID-19 patients[59]. In a later report, moderate COVID-19 patients treated with tocilizumab showed fewer severe infections than those who received a placebo. However, tocilizumab did not prevent the need for intubation or death in these patients[60]. Thus, the role of tocilizumab in the treatment of COVID-19 remains obscure.

Encouragingly, a meta-analysis of seven randomised clinical trials revealed lower 28-day mortality among critically ill patients who received systemic corticosteroids compared to those who received usual care or placebos[61]. In the RECOVERY trial, the immunosuppressant dexamethasone (6 mg once daily for up to 10 days) reduced 28-day mortality in patients who required oxygen, particularly in those receiving mechanical ventilation[47]. No benefit was found for patients who did not require oxygen supplementation[47]. The mechanism that underlies the beneficial effect of dexamethasone is not well understood in these patients. It may be associated with the inhibition of major pro-inflammatory pathways, such as NF-κB, in the most severe patients (Fig. 1a and 1f)[38]. Nonetheless, these clinical trials suggest that cytokine storms contribute to lung injury and multi-organ failure in severe COVID-19 patients. For this reason, major health

organisations recommend dexamethasone (or potentially other glucocorticoids) as standard care for patients with severe COVID-19.

Other factors that influence the infection cycle.—CoV-2 infection cycles are associated with diverse clinical characteristics in COVID-19 patients, manifesting no, mild, or severe symptoms, such as acute respiratory disease and pneumonia[62–64]. Evidently, some asymptomatic patients have persistent negative computed tomographic findings[65], suggesting low viral load or low inflammatory and immunological responses in the lungs. Approximately 80% of the infections are asymptomatic or mild, 15% are severe (requiring oxygen inhale), and 5% of patients are in critical conditions and require a ventilator[66]. At this time, it is impossible to predict which patient will become one of that 5% to need critical care.

Many tangible intrinsic (such as age, gender and ethnicity) and extrinsic factors (such as lifestyle) influence the infection cycle, morbidity and mortality rates. CoV-2 infects humans from neonates to older adults[67–69]. However, paediatric cases are less often symptomatic than older adults[69–71]. CoV-2 infection also affects women less than men[72], possibly because androgen signalling modulates ACE2 levels. Increased androgen levels are associated with a higher risk of CoV-2 infection and disease severity in men[17]. Demographic data reveal high morbidity and mortality rates in African Americans in the USA[73–75], although underlying reasons remain unclear and could likely be multifactorial, including socioeconomic factors and access to healthcare.

Cigarette smoking increases susceptibility to CoV-2 infections by upregulating *ACE2* expression[16,76,77]. Collectively, age, gender, lifestyle, and demographic differences might modulate viral receptor expression and other unknown determinants, which, in turn, contribute to disease severity and therapeutic response. For instance, co-expression of *ACE2* and *TMPRSS2* mRNAs is tightly regulated in an age- and gender-dependent manner and upregulated in individuals who smoke[78].

In summary, age, gender, and genetic background will have to be integrated into conventional CoV-2 assays and COVID-19 models to facilitate the screening of antiviral drugs and antibodies and predict therapeutic responses. Conventional COVID-19 assays can be classified into three categories: *in vitro* biochemical, pseudotyped and live virus assays (Fig. 2a). In this Review, we would like to focus on cell culture models for COVID-19 research (Fig. 2b) and refer the reader to related reports and excellent Reviews on conventional assays[79–85].

Cell culture models for COVID-19 research

Theoretically, all cellular processes of the CoV-2 infection cycle could be used for assays to examine CoV-2 infectivity and for drug screens. At present, several experimental platforms and cell types exist for clinical and experimental coronavirus research, all of which have benefits and limitations (Fig. 2b). Among them, we focus on the three major systems used to study COVID-19: 2D monolayer cell culture, adapted 2D air-liquid interface (ALI) methods, and 3D culture or organoids (Fig. 2b).

2D monolayer culture.—2D monolayer cell cultures (Fig. 2b2) of various cell lines, such as 293T, A549, BHK, Caco-2, MDBK, PK-15, and Vero cells (available from the American Type Culture Collection) have been used to determine CoV-2 cell entry and for drug testing[41,86]. TMPRSS2-expressing Vero-E6 cells, which have a similar ACE2 structure to that of human cells, are highly susceptible to CoV-2 infection[14,87] and represent an effective culture method to propagate CoV-2 and measure the viral load of CoV-2 variations.

ALI assays.—The ALI culture mimics the *in vivo* airway environment and is widely used in the maturation and functional assessment of the airway epithelium[88]. ALI assays allow the apical side of the epithelium to contact the air and the basolateral side to access the differentiation medium through a microporous membrane (Fig. 2b3). 2D-ALI is particularly suitable to evaluate links between air-borne related lung pathologies and susceptibility to a severe CoV-2 infection[16]. Limitations of this method are an inability to passage the culture, which means that it cannot be scaled up and used in high throughput assays, and its inability to generate more complex tissue structures, such as alveoli. Historically, growth and differentiation of respiratory basal cells in an ALI culture has been challenging in the absence of non-basal cells. For example, KRT5-GFP$^+$ basal cells of the mouse trachea require a 500-fold excess of non-basal cells in ALI experiments to achieve approximately 6% colony-forming efficiency (based on counting large colonies) at day 21[89]. Using an adapted 3D sphere-forming assay, Hogan and colleagues were able to seed single KRT5-GFP$^+$ basal cells of the mouse trachea in the absence of stroma or non-basal cells[90]. This 3D-culture adaptation leads to a rapid formation of “tracheospheres” within one week and a sphere-forming efficiency that is comparable to ALI experiments described above[89,90].

3D cell culture and organoids.—Unlike 2D, 3D cell culture is an artificially created platform that mimics the *in vivo* environment of living cells and tissues. Cells are usually grown in suspension in suitable medium or extracellular matrices (such as Matrigel and collagen) to form spheroids or 3D colonies. The extracellular matrix components and physical forces play a vital role in regulating cell behaviour. Current organoid protocols partially recapitulate 3D cellular environments *in vivo* and retain the genetic and epigenetic features of human cells. They can be long-term expanded, banked for personalised medicine (Fig. 2b4) and used to model viral infectious diseases[6–9,91,92]. 3D organoids can be dissociated and adapted to 2D ALI cultures to facilitate directed differentiation of airway stem/progenitor cells into mature cells for downstream assays[93,94] and apical viral respiratory infection[94]. An overview of the strengths and limitations of 2D and 3D culture is available in Figure 2b and previous Reviews[6,8,95]. Taken together, organoids represent a powerful platform for COVID-19 research.

hPSC-derived organoids for COVID-19 research

Organoids can be derived from hESCs or hiPSCs (we use the term hPSC for both) and maintained as a 3D tissue *in vitro*, capable of self-organising and self-renewal. They have been successfully used for disease modelling and drug discovery[6,7], thus paving the way to study COVID-19 *in vitro*.

Modelling COVID-19.—The first proof-of-concept experiment demonstrated that CoV-2 infects human blood-vessel and kidney organoids[14], which can be blocked with human recombinant ACE2[14]. Subsequent reports confirmed that diverse types of hPSC-derived organoids, including intestinal, cardiac, brain, choroid plexus, and lung organoids, can be used as disease models to study the tropism of CoV-2 and for drug screening[10,12,15,17,18]. Lung organoids are particularly suitable as epithelial cells of the respiratory airways and alveoli are both targets and effectors of CoV-2 infections (Fig. 3).

Lung organoids.—The human lung is a complex organ with highly branched and progressively thinner tubes that carry air into the distal alveolar sacs. It comprises multiple integrated compartments: proximal and intermediate airways, respiratory bronchioles, and alveoli (Fig. 3a, 3b)[96]. Each compartment is populated by various cell types, including epithelial, vascular, stromal, and immune cells (Fig. 3b, 3e)[78,97–99]. The intermediate airways have a pseudostratified epithelial layer that holds heterogeneous cell types, including secretory Club cells, multiciliated cells, mucus-producing goblet cells, transient secretory cells and basal (stem) cells (Fig. 3b, 3e). The distal respiratory bronchioles are lined with a poorly characterized cuboidal epithelium. The alveoli are covered by alveolar epithelial type 1 and 2 cells (AECIs and AECIIs), important for gas exchange and alveolar homeostasis. Each compartment also has its own stem/progenitor population with specialised functions in response to environmental insults (Fig. 3b, 3e).

Airway epithelial cells are generated from hPSCs by imitating multi-stage lung developmental trajectories[100], for instance to derive lung bud organoids that recapitulate lung development and disease[101,102]. Lung organoids containing more mature epithelial cells have also been created from hPSCs *in vitro*[10,17,103–106]. The derivation of lung organoids varies from protocol to protocol. However, the major consensus steps may be summarised, based on a well-documented protocol[107], as follows. First, definitive endoderm is induced from hPSCs by Activin. Second, anterior foregut endoderm and foregut spheroids are sequentially formed by inhibiting BMP4, TGF-β, and GSK3β, in the presence of FGF4 and Smoothened agonist. Third, bud tip progenitor organoids are induced by FGF7, ATRA, and GSK3β inhibition. Finally, complex lung organoids containing airway-like structures, mesenchymal-like cells, and alveolar progenitors are obtained *via* a prolonged incubation with foetal bovine serum and FGF10 (Fig. 3c).

Lung organoids are classified into bronchospheres, bronchioalveolar organoids, and alveolospheres[98,108] (Fig. 3d). In bronchospheres, secretory Club cells and basal cells represent stem-cell-like cells. Secretory Club cells are a CoV-2 target as they co-express the highest levels of *ACE2* and *TMPRSS2*, compared to basal, ciliated, and alveolar cells in the lung (Fig. 3f)[109]. *ACE2*, *TMPRSS2*, and *FURIN* are also co-expressed in bronchial transient secretory cells that show high Rho GTPase activity and viral processes related to membrane remodelling or the immune system[78], likely underlying their vulnerability to CoV-2 infection.

Alveolospheres contain flat AECIs and cuboidal AECIIs (Fig. 3d and 3e). AECIIs function as stem/progenitor cells in the adult lungs[110], co-express *ACE2* and *TMPRSS2*, and serve as another major CoV-2 target[111,112]. Not surprisingly, CoV-2 heavily affects alveolar

pneumocytes (including AECII cells) in COVID-19 patients, leading to diffuse alveolar damage, respiratory failure, and increased mortality[39,40,113,114]. For this reason, hPSC-derived lung organoids should be particularly useful for the study of severe COVID-19.

Drug discovery.—hPSC-derived alveolar organoids have been used in CoV-2 infection assays, high-throughput drug screens, and drug repurposing[10,17,115–118]. For example, androgen receptor signalling inhibitors finasteride and dutasteride reduced the infectivity of CoV-2 in hESC-derived lung alveolar organoids by lowering ACE2 and TMPRSS2 levels[17]. A high-throughput drug repurposing screen in organoids identified multiple compounds (imatinib, mycophenolic acid, quinacrine dihydrochloride) that inhibit the cell entry of CoV-2[10]. Similarly, an ACE2 blocking antibody inhibited viral entry in an organoid model, enhanced the activity of M2 macrophages, and suppressed pro-inflammatory effects mediated by M1 macrophages[118]. These *in vitro* experiments confirm that alveolar precursors and differentiated AECIIs are permissive to CoV-2 infection, elicit a cytokine response and can be used to identify compounds that block CoV-2 infection.

Despite these promising initial results, organoid models also have a number of limitations that should be considered. For instance, hPSCs are prone to genomic instability in long-term *in vitro* culture[119–123]. Further, inter-laboratory protocol differences inevitably increase experimental variability and cell culture and differentiation protocols are inherently time-consuming[107,124]. Finally, immature differentiation of lung organoids under suboptimal culture conditions remains a frequently encountered and unresolved issue[125].

Human lung organoids often produce developmentally immature foetal lung tissues with a higher proliferation rate *in vitro*[101,105,117,126]. Epithelial cells from hESC-derived organoids express precursor markers such as NKX2.1 and SOX9[17]. As a partial solution, 3D-organoid-converted 2D ALI cultures are increasingly used to enhance the maturity of differentiated respiratory epithelial cells for downstream analysis[116,117,127]. Nonetheless, a deeper understanding of the developmental principles underlying cell maturation and niche environments is necessary to optimise organoid protocols. These insights could facilitate the creation of chemically defined media and improved extracellular matrices or scaffolds[124,128,129].

In summary, hPSC-based organoids are valuable for personalised medicine and disease modelling. They provide excellent platforms for drug efficacy and drug repurposing studies[10,17,115]. The expression of multiple CoV-2 susceptible genes in lung organoids makes them ideal models to study infectivity. However, we recommend verifying the results obtained from hPSC-derived organoids in animal models and organoids established from human ASCs.

ASC-derived organoids for COVID-19 research

The definition of ASCs varies in the scientific literature due to the complexity of cellular properties, including cellular dynamics[130], heterogeneity[131], and plasticity[132]. In addition, it can be difficult to distinguish ASCs from progenitor cells. In this Review, ASCs are defined as rare, mostly quiescent, and multipotent cells found in adult tissues. They are capable of long-term self-renewal, generate intermediate cell types (progenitors) with limited self-

renewal potential, and differentiate into tissue-specific cells[7,97]. ASCs can be isolated from the adult issue and maintained in cell culture indefinitely if supplemented with appropriate microenvironments and growth factors. ASCs and progenitors serve as valuable alternatives to hPSCs, providing a source of fully mature cells for functional analysis.

Intestinal and nasal organoids.—Intestinal organoids and nasal spheroids have been derived from donor biopsies and were previously used to predict drug responses in patients with cystic fibrosis[95]. Differentiated enterocytes express *ACE2* and *TMPRSS2* (Fig. 4a) and substantial titres of CoV-2 particles were also detected in enterocytes of intestinal organoids[13]. Transcriptomic analysis indicated a strong viral response with enrichment of *CXCL10* and *CXCL11* mRNAs[13], closely related to a cytokine storm. This study supports that ASC organoids can be used to study CoV-2 pathophysiology *in vitro*.

Interestingly, the nasal mucosa also highly co-expresses *ACE2* and *TMPRSS2* (Fig. 4a)[109], consistent with the heavy CoV-2 particle load in the nasal cavity of COVID-19 patients. The nasal mucosa has a similar epithelial lining to that of the upper respiratory airway, including secretory Club cells and basal stem cells[109]. As nasal biopsies are minimally invasive compared to intestinal or lung biopsies, nasal spheroids provide a valuable resource and surrogate for lung organoids.

Lung organoids.—Evidence suggests that both ASC-like cells and progenitors exist in different compartments of the lungs. Basal cells in the intermediate airways meet the definition of generic ASCs[90,94]. Basal stem cell organoids contain basal cells, secretory goblet cells, and ciliated cells (Fig. 3d, 3e). Airway basal stem cells have been isolated from human biopsies and expanded for functional assays of the airway repair response after CoV-2 infection[16].

ASC-like cells or progenitors were also found within the SCGB1A1$^+$ secretory Club and AXIN2$^+$ AECII cell populations in human adult lungs (Fig. 3b, 3e)[96,108]. Mouse genetic lineage analysis revealed that surfactant protein C positive (SFTPC$^+$) AECII cells in the alveolar niche are ASC-like cells, giving rise to self-renewing “alveolospheres” that contained both AECII and AECI-like cells[110]. In mice, rare Axin2$^+$ AECIIs also act as alveolar stem cells and secrete Wnt molecules to recruit “bulk” AECIIs as the progenitors[133]. A distinct population of mouse IL1R1$^+$ AECIIs can become damage-associated transient progenitors (DATPs), which then differentiate into mature AECIs[134]. In mouse and human lungs, similar alveolar epithelial progenitors (AEPs) reside within the AECII pool and generate mature AECIs and AECIIs from alveolar organoids[135]. Thus, AECIIs constitute an important stem/progenitor source in the alveoli.

Human alveolar organoids have been derived from adult AECIIs to assess CoV-2 infection[11,112,127,136,137]. These *in vitro* experiments confirm that AECIIs are the principal target of CoV-2. CoV-2-infected alveolar organoids mirror many features of COVID-19 patients, including cytokine release, interferon (IFN) and immune response, loss of surfactant proteins, and cell death. AECII-based organoids, derived in a feeder-free and chemically defined culture system, could be sustained long-term[112] and revealed that few (≥1) CoV-2 particles that enter alveolar cells can lead to a full infection. Genes associated

with cell death, cell adhesion, and surfactant proteins were also upregulated in CoV-2 infected AECIIs[112].

IFN-mediated inflammatory signalling is a typical response to CoV-2 infection documented in these studies. An increase in the IFN response was associated with a lower CoV-2 burden (around 60 hours post CoV-2 infection of alveolar organoids) and vice versa for a decrease in the IFN response[112]. Pretreatment of alveolar organoids with low dose IFN-α and IFN-γ reduced CoV-2 replication[11]. In contrast, IFN inhibition endorsed viral replication[11]. Pretreatment of alveolar organoids with IFNB1 (INF-β) also reduced expression of the viral RNA gene *N*, which encodes the CoV-2 nucleoprotein[127]. These findings suggest that the administration of IFNs may be a possible prophylactic measure against severe CoV-2 infection.

Pharmacological inhibition of CoV-2 infection with small molecules is of considerable interest. ASC-derived lung organoids have not yet been used for drug repurposing or discovery studies. However, a study confirmed that remdesivir decreases CoV-2 *N* gene expression more effectively than INF-β or hydroxychloroquine in infected alveolar organoids[127]. This finding is intriguing but contradicts the ineffectiveness of remdesivir in the recent clinical trial discussed above[48]. Inconsistencies between the results from alveolar organoids and the clinical trial need to be further investigated.

In summary, lung organoids derived from adult human lungs generate respiratory epithelial cells with high maturity compared to hPSC-derived organoids and are suitable for studying COVID-19. However, it is often difficult to obtain lung tissues with the desired quality and materials are scarce, as samples are typically acquired from bronchioalveolar washings and lung explants with institutional review board approval[108]. In contrast to hPSC-derived lung organoids, ASC- or progenitor-based lung organoids exhibit limited self-renewal capacity, usually less than five passages. As for the former, developmental paradigms can guide the derivation of long-term expandable lung organoids from the adult lung[94]. For instance, FGF7 and FGF10 are vital in establishing long-term expandable lung organoids from adult tissues[94,138]. Interestingly, both factors are also required in the final steps to generate hPSC-derived lung organoids (Fig. 3c)[107].

Improving lung regeneration.—Little is known about the regenerative capacity of the alveolus in COVID-19 patients. Organoid studies revealed that CoV-2 infected AECIIs exhibit defence and repair mechanisms to combat injury, such as cytokine secretion, resistance to apoptosis, and cellular senescence[11,112,118,139]. AECIIs still proliferate, transit to different cellular states, and differentiate into AECI-like cells. These cellular processes closely mimic regenerative responses in mouse and human injury models[110,133–135]. Further support for a targeted regenerative response comes from a study demonstrating that AECIIs proliferate and differentiate into squamous AECIs in severely affected alveoli of COVID-19 patients[40].

Despite the existence of endogenous repair mechanisms, a number of recovered COVID-19 patients will require therapy to restore the lost lung function and repair the damage to alveolar cells. Replacing damaged alveolar cells with a suitable AECII source might be a

possible way to improve lung function. Encouragingly, mature AECIIs of both mouse and human origins can be transplanted into injured mouse lungs[140,141]. Vunjak-Novakovic and colleagues proposed an airway-specific method to de-epithelialize the distal lung airways and preserve the basement membrane and vascular endothelium. This approach enabled the functional vascularisation of lung grafts to support the attachment and growth of hiPSC-derived epithelial cells in a rat model[142]. Similar transplantation approaches using organoid-derived lung epithelial cells may be applicable for treating COVID-19 patients with the severe epithelial injury in the future. Still, many preclinical challenges remain to be overcome, most notably relating to source cell identity, immunological compatibility, and functional integration into the host.

Opportunities and challenges

CoV-2 infection does not only affect the lung but can damage any cell that expresses *ACE2* or co-expresses *ACE2* and *TMPRSS2* (Fig. 4a). The ACE2 receptor, initially identified as a cardiac regulator, is present on oral mucosa, AECII pneumocytes, intestinal, kidney, cardiac, smooth muscle and endothelial cells[143–146]. Transcriptomic profiling provides a comprehensive view of *ACE2* and *TMPRSS2* expression in cells of the human body[109]. These datasets are particularly helpful when choosing specific organoids for COVID-19 research (Fig. 4a).

Established cell lines and organoids.—Currently, hiPSC-derived patient-specific lung organoids recapitulate the pathophysiology of various lung diseases, such as surfactant deficiency[147], cystic fibrosis[106], Hermansky–Pudlak syndrome[101,148], and respiratory syncytial and parainfluenza virus infection[101,102]. In another example, AECIIs, derived from a child with a lethal neonatal respiratory distress syndrome caused by a homozygous SFTPB mutation, mimicked aspects of this syndrome, including the deficiency in surfactant processing[147]. The abnormal processing could be restored in gene-corrected AECIIs from this patient[147]. So far, experiments with hiPSC-derived organoids have confirmed that lung epithelial cells are susceptible to CoV-2 infection, express CoV-2 host factors, and provoke an intrinsic epithelial inflammatory response[116,117,126]. However, these hiPSC-derived lung organoids have not yet been used to unveil the differences associated with inherent variations to infection and immune activation in large COVID-19 patient cohorts[116,117,126]. Organoids derived from established hiPSC lines and cell banks from individual donors or patient biopsies provide a valuable and convenient tool to assess risk factors and therapeutic outcomes in the most vulnerable populations and implement targeted prevention at low cost. Currently, lung, cardiac, intestinal, liver, kidney, and capillary organoids are immediately available to serve these purposes.

Complex organoid assays.—One major remaining challenge in the application of organoids is the lack of complexity and extensive intercellular interactions compared to the *in vivo* situation. Complex lung airway organoids can be derived by integrating human adult primary bronchial epithelial cells and lung fibroblasts with lung microvascular endothelial cells to study disease-relevant cell-cell interactions[149]. However, hPSC-derived alveolospheres typically have few cell types (for instance AECIs and AECIIs) and do not include adjacent capillaries composed of endothelial cells (Fig. 3b).

The endothelium is connected with alveolar cells by the basement membrane and acts as alveolar niche. Mouse alveolospheres form more easily in organoid co-cultures with lung endothelial cells[150]. Successful alveolar repair requires restoration of the spatial relationship between alveolar cells and the endothelium. Endothelial cells can receive reparative signals from AECIs after acute injury[151] and enhance alveologenesis through diverse signalling factors such as endothelial-derived angiocrine factors and platelet-derived SDF-1[152,153]. Endothelial cells also secrete the vascular endothelial growth factor, monocyte chemoattractant protein–1, IL-6, and IL-8, all of which aggravate the cytokine storm[35]. Reduced vascular endothelial cadherin expression on endothelial cells[154] increases vascular permeability and pulmonary dysfunction in ARDS. For these reasons, vascularised lung organoids should provide valuable insights into CoV-2-mediated lung damage and repair.

So far, the generation of vascularised lung organoids has not been achieved, although vascularisation occurs in lung organoids grafted into animals to promote cell differentiation[101,129]. For the liver, vascularised organoids termed liver buds have been reported, which consist of hiPSC-derived hepatic endoderm, endothelial cells, and bone marrow stromal cells[155]. Similarly, the integration of vasculature into cerebral organoids seems to accelerate functional maturation of neurons[156]. It seems likely that vascularised lung organoids might exhibit comparable properties. At any rate, improved protocols for complex organoids with integrated capillary systems are expected to more accurately recapitulate the alveolar CoV-2 response (Fig. 4b2).

Another major challenge is the lack of host factors, immune cells and inflammatory responses in existing organoid assays[66,157]. Drug and antibody effects observed in these assays may not reflect genuine responses of COVID-19 patients, who often have additional medical conditions such as cardiovascular disorders or diabetes that alter cellular behaviour[158]. Ideally, organoids should include multiple cell types to closely mimic *in vivo* environments and the complexity of hyper-inflammatory complications in patients (Fig. 4b4). We previously proposed a concept that encompasses the multi-dimensionality of organoids[6]. In addition to 3D structures, we propose that 4D organoids emphasize the developmental time scale in an organoid culture. By comparison, 5D organoids would further integrate extrinsic factors to simulate host environmental, immunological, and inflammatory signals, which are absent from current organoid cultures[6]. Highly sensitive ELISA-based analysis of the cell culture medium is essential for monitoring cytokine storm inhibition in these complex organoids.

In the future, organoids could also have a role in vaccine development. Traditional vaccine development is a long-lasting process. Exploratory efforts on vaccine design, preclinical evaluation in animal models and clinical phase I, II, and III trials can take 15 years or more[159]. Although organoids may help identify potential molecular targets for vaccine design, they cannot be directly used to derive a vaccine due to the absence of the host immune system. However, organoid assays may provide valuable information about intermolecular interactions between viral proteins and host receptors and determine the efficacy of neutralising antibodies from vaccinated individuals (Fig. 2). In this way, organoid platforms might accelerate vaccine development at all stages that involve exploratory work, preclinical evaluation, and the assessment of efficacy in clinical trials. Encouragingly, the

generation of immune cell organoids (for instance, T-cell and B-cell specific organoids) is feasible from hiPSCs[160]. The presence of T cells, B cells, and macrophages in organoid systems might well improve vaccine assessment in the future (Fig. 4b4).

Importantly, the integration of multiple cell types is limited by developmental constraints for a specific organ in a defined niche environment. As with all other organoid systems, a thorough understanding of the underlying developmental biology will be required for further progress in the creation of complex organoids.

Concluding Remarks

The inherent physiological variability of human populations poses a major challenge for the assessment of individual susceptibility and therapeutic outcomes. The versatility of hPSC-based and ASC-based organoids makes them a useful platform to compensate for the shortcomings of current assays. Multi-tissue isogenic organoids from individual donors and patients enable robust molecular assessment of the vulnerability of individual patients and possibly predict therapeutic responses in severe COVID-19 cases. We envision that the combination of current assays with complex organoids will continue to improve COVID-19 research and treatment, and provide valuable lessons for the study of other viral diseases as well.

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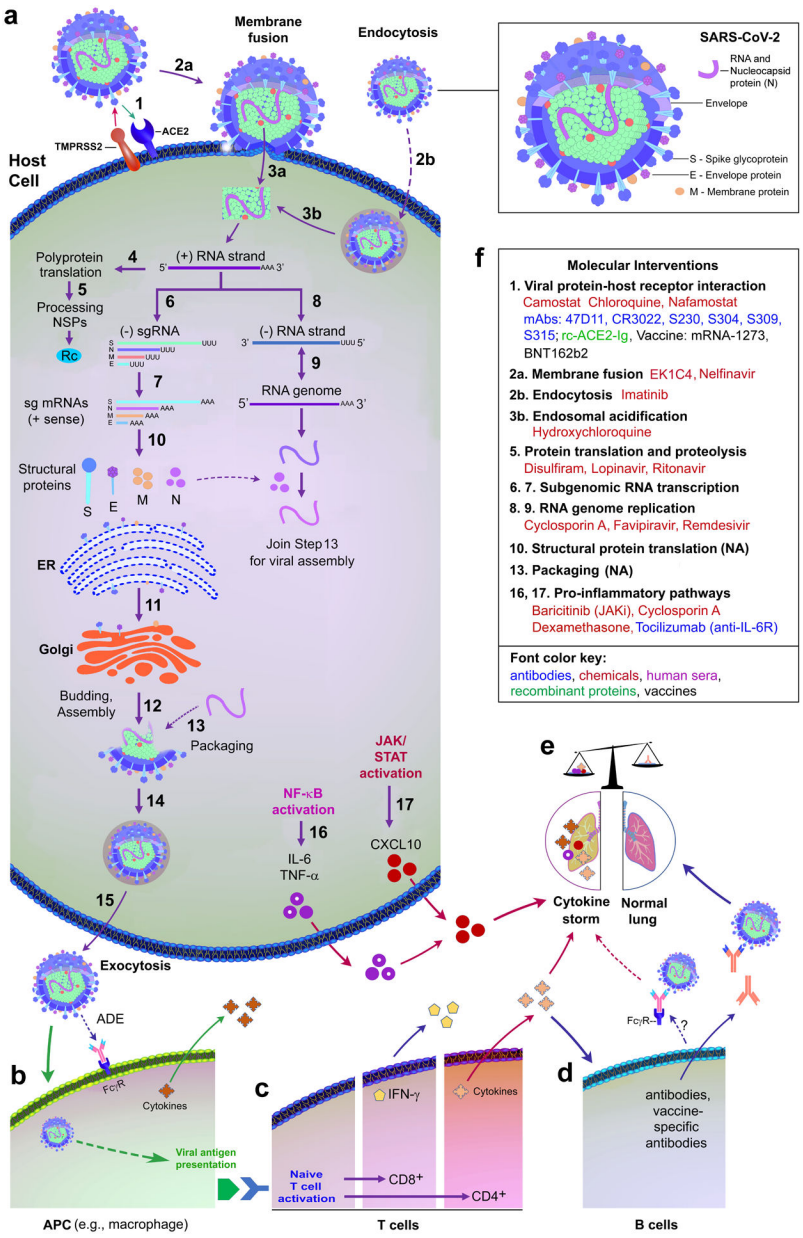

Fig. 1: SARS-CoV-2 (CoV-2) infection cycle, immunological response, molecular targets, and intervention strategies.

(**a**) The infection cycle includes spike glycoprotein (S-gp) binding to the human angiotensin-converting enzyme 2 (ACE2) receptor, pre-cleavage by the host cellular protease furin to dissociate the S1 subunit from the S2 subunit of S-gp[161,162], and S2 activation mediated by serine protease TMPRSS2 co-receptor[41]. Notably, cleavage by furin is required for the entry of CoV-2 into human lung cells[161]. S2 activation triggers viral and host cell membrane fusion. Within the host cell cytoplasm, the positive-sense CoV-2 genomic RNA is transcribed to yield full-length negative-sense RNAs (for genome replication) and subgenomic negative-sense RNAs (-sgRNA, for producing subgenomic mRNAs). Subgenomic mRNAs, converted from -sgRNAs, are translated into viral structural proteins, including S-gp, envelope (E), membrane (M), and nucleocapsid (N) proteins[30–32]. Finally,

viral genome encapsulation and reassembly enable virus maturation and export out of cells for the next infection cycle. (**b** and **c**) CoV-2 induces immunological responses through viral antigen presentation in macrophages, naive T cell activation, and release of cytokines. (**d**) A possible dual role of B-cell-mediated humoral immune response: B cells generate the neutralising antibodies to protect the lung from CoV-2 infection and contribute to cytokine-induced damage through FcγR-mediated and antibody-dependent enhancement of CoV-2 infection. (**e**) CoV-2-induced organ damage via an unbalanced presence of pro-inflammatory cytokines or absence of antiviral factors. (**f**) Representative intervention strategies, such as the development of drugs, vaccines, antibodies, recombinant proteins and repurposing of approved drugs against CoV-2 infection, based on molecular targets in Figure 1a. Abbreviations: ACE2, Angiotensin-converting enzyme 2; ADE, antibody-dependent enhancement; APC, antigen-presenting cells; CXCL10, C-X-C motif chemokine ligand 10; ER, endoplasmic reticulum; FcγR, Fc-gamma receptor; IFN, interferon; IL-6, interleukin 6; IL-6R, Interleukin 6 receptor; JAK, Janus kinase; JAKi, Janus kinase inhibitor; mAbs, monoclonal antibodies; NA, data not available; NF-κB, nuclear factor kappa B; Rc, replicase and transcriptase complex; NSPs, non-structural proteins; rc-ACE2-Ig, recombinant ACE2-Ig; STAT, signal transducer and activator of transcription; TMPRSS2, transmembrane protease serine 2; TNF-α, tumour necrosis factor alpha.

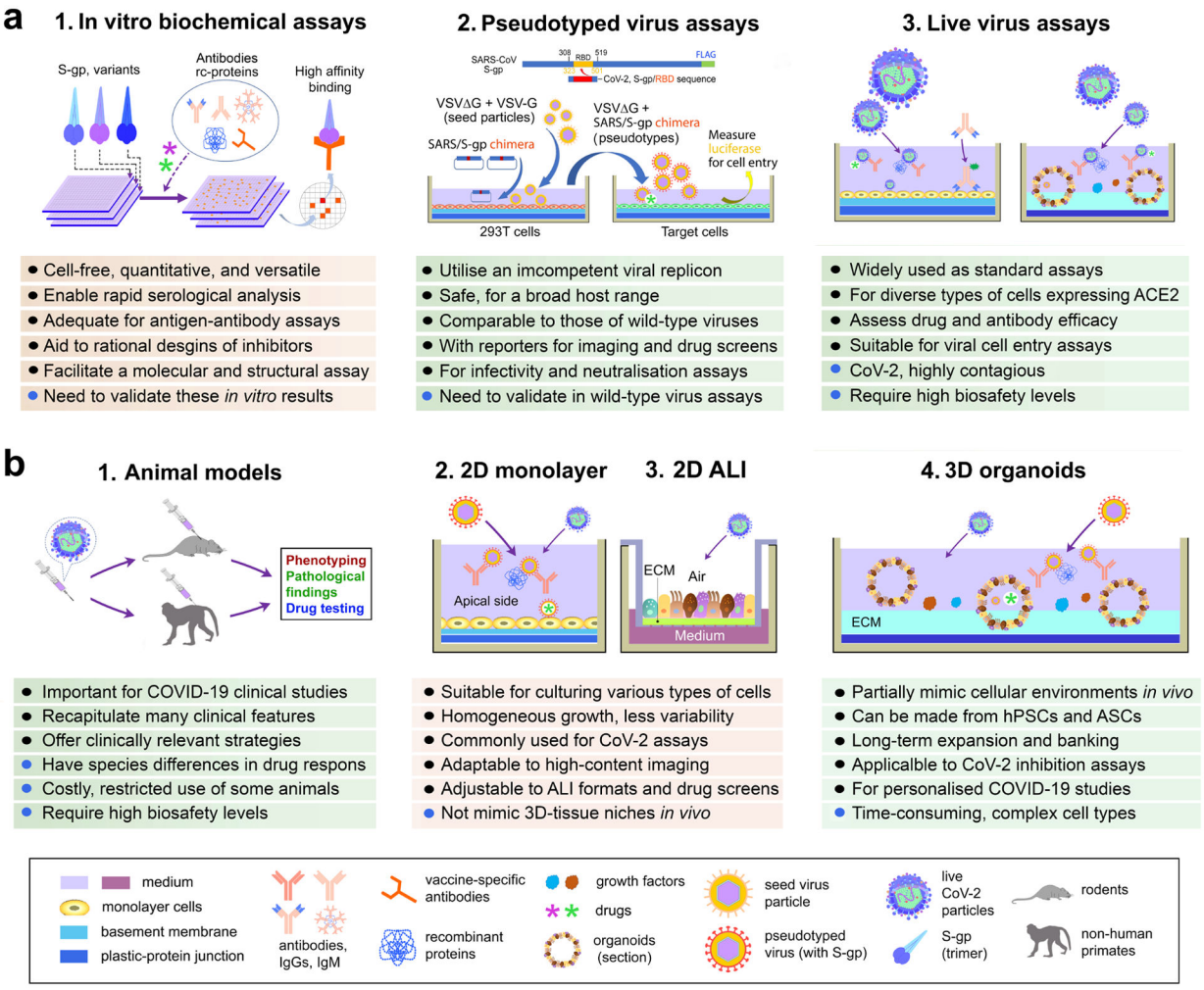

Fig. 2: COVID-19-related assays.

(**a**) Assays are categorised as *in vitro* cell-free molecular and biochemical, pseudotyped virus, and live virus assays. Pseudotyped virus experiments are exemplified by pseudotyped VSV harbouring VSV-G and a SARS-CoV-S-gp chimeras. At 16-hour post inoculation, the pseudotyped viral entry is analysed by determining luciferase activity in cell lysates[86]. No envelope glycoprotein pseudo-viral control is used for normalization. (**b**) Assays can be animal models, 2D-monolayer cell culture, 2D air-liquid interface (ALI) transwell culture, and 3D organoids. The combination of platforms empowers the utility of these assays for Covid-19 drug and vaccine development. Abbreviations: ASCs, adult stem cells; CoV2, SARS-CoV-2; ECM, extracellular matrix; hPSCs, human pluripotent stem cells; rc-proteins, recombinant proteins; S-gp, Spike glycoprotein; VSV-G, wild-type vesicular stomatitis virus; VSVΔG, vesicular stomatitis virus with deletion of the envelope glycoprotein (G).

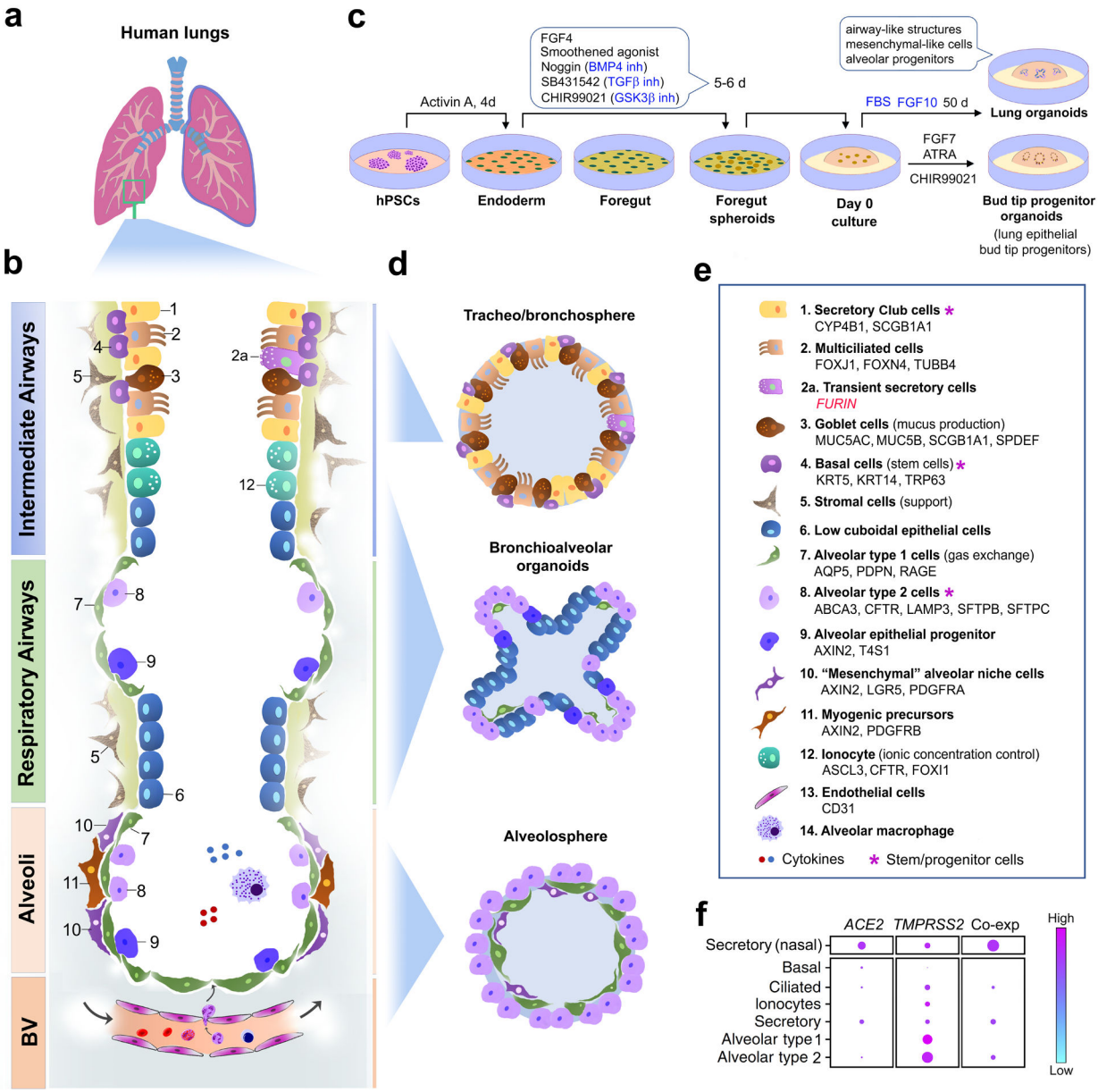

Fig. 3: Lung cell types and organoids.

(**a**) Human lung anatomy. (**b**) Schematic of the major cell types in different compartments of the human lung, partially adapted from references[78,97–99]. (**c**) A representative protocol for the generation of lung organoids containing cell types of interest[107]. (**d**) Schematic of lung organoids that model different cellular compartments of the lung. (**e**) Cell types in panels b and d, with gene and protein markers listed alphabetically[78,97–99,163,164]. (**f**) Representative single-cell RNA sequencing analysis of CoV-2 receptor gene expression and co-expression (co-exp)[109] in major cell types of the respiratory airways and alveoli. Nasal secretory cells are used as control for comparison. The size of the dots is proportional to the percentage of cells that express indicated genes (adapted from the published data in reference[109]). Abbreviations: ABCA3, ATP-binding cassette subfamily A member 3; ACE2, angiotensin-converting enzyme 2; AQP5, aquaporin 5; ASCL3, Achaete-Scute family BHLH

transcription factor 3; ATRA, all-trans retinoic acid; BMP4, bone morphogenetic protein 4; BV, blood vessels; CFTR, CF transmembrane conductance regulator; CYP4B1, cytochrome P450 family 4 subfamily B member 1; d, day(s); FBS, foetal bovine serum; FGF, fibroblast growth factor; FOXI1, forkhead box I1; FOXJ1, forkhead box J1; FOXN4, forkhead box N4; GSKβ, glycogen synthase kinase 3 beta; hiPSC, human induced pluripotent stem cells; inh, inhibitor; KRT5/14, keratin 5/14; LAMP3, lysosomal associated membrane protein 3; LGR5, leucine-rich repeat-containing G-protein coupled receptor 5; PDGFRA/B, platelet derived growth factor receptor alpha/beta; PDPN, podoplanin; SCGB1A1, secretoglobin family 1A member 1; SFTPB/C, surfactant protein B/C; SPDEF, SAM pointed domain containing ETS transcription factor; TMPRSS2, transmembrane serine protease 2; TUBB4, tubulin beta 4B class IVb.

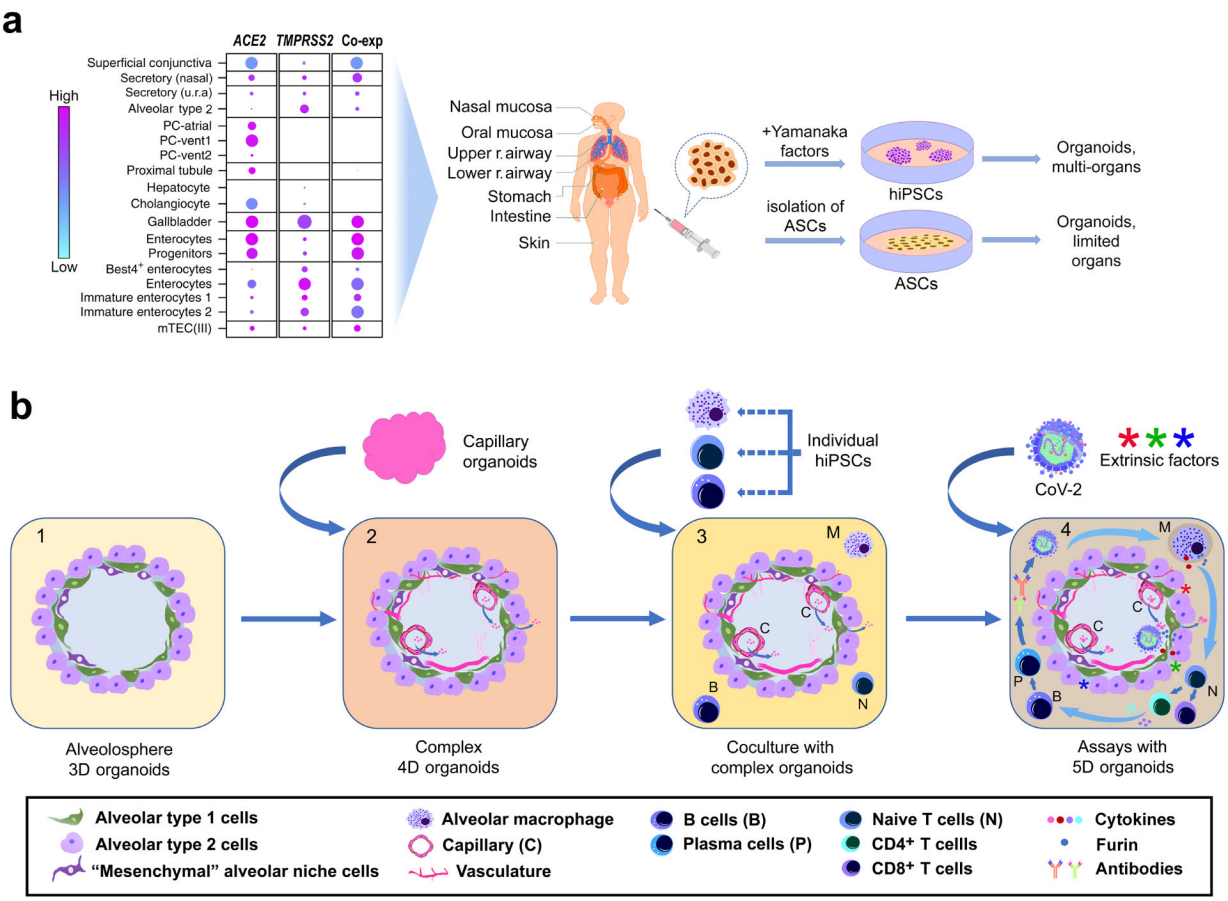

Fig. 4: Stem-cell-based organoids to assess SARS-CoV-2 (CoV-2) susceptibility.

(**a**) CoV-2 receptor gene expression and coexpression (co-exp) in human cells (adapted from reference[109]). Isogenic organoids can be generated from adult stem cells (ASCs) and human induced pluripotent stem cells (hiPSCs). The size of the dots in the left panel is proportional to the percentage of cells that express the indicated genes. (**b**) Development of multi-dimensional organoids to model the complexity of immunological and hyper-inflammatory complications in COVID-19 patients. Abbreviations: mTEC (III), medullary thymic epithelial cells of the foetal thymus; PC-atrial and PC-vent, pericytes in the atrium and ventricle of the heart; r. respiratory; secretory (u.r.a), secretory cells from the upper respiratory airway.