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How to optimize respiratory models for SARS-CoV-2 research

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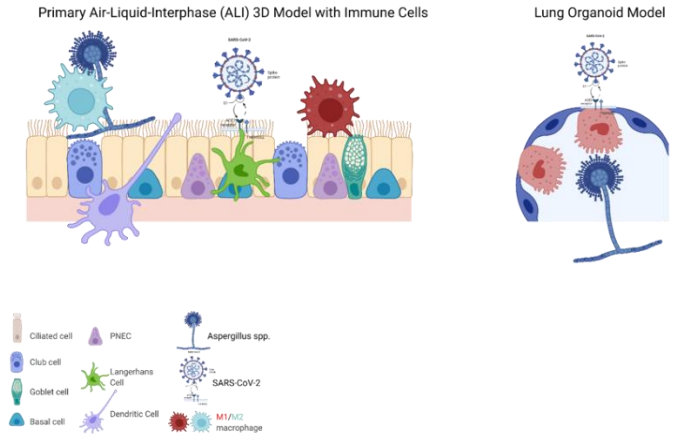
Abstract



Sophisticated 3D cell culture tissue models experienced a boom in the last years and in particular human cell culture and 3D respiratory systems greatly supported the development of novel drugs and vaccines during the SARS-CoV-2 pandemic lately. These models provide multiple benefits in terms of similarities in differentiation, metabolism, receptor expression, polarity, infectivity compared to human tissues and thus provide excellent models to study very first interactions with the host during pathogen entry. Dependent on the experimental approach, the use of different 3D models is more beneficial – apical out lung organoids for e.g., high content screening (HCS) of treatment options, air-liquid interphase (ALI) models for e.g., easy incorporation of immune cells, screening of epithelial integrity or mucociliary clearance. This review will give an overview on the models established in our laboratory and on their applications.

1. Introduction

The use of organotypic three-dimensional (3D) cell cultures in basic, translational and clinical research is now more popular than ever - rapid development is taking place in this area due to modern technologies, availability of induced pluripotent stem cells (iPSCs), commercially available and more physiologically relevant primary cells or biobanking- opportunities.

Often, these novel approaches use self-assembling 3D organoid or spheroid cultures, which offer several advantages over conventional 2D cell cultures. In addition to 3D organoid/spheroid cultures, there are other options for 3D cultivation, such as scaffold-based methods and long-term differentiation within an air-liquid interphase (ALI) for e.g., respiratory tissues to mimic the situation in the respiratory tract. 3D culture technologies offer a great potential for a more realistic disease modeling *in vitro* or for testing drugs in a personalized way. The simulation of tissues in cell cultures offers the opportunity for answering scientific questions that cannot be met using conventional 2D cell culture monolayers or animal experiments. Thus, we will here review current progress of respiratory tissue model optimization performed in our lab during the last 10 years and will highlight our recent findings on pathogen-barrier interactions using a viral (SARS-CoV-2) challenge.

2. Respiratory 3D models and their applications

Dependent on the experimental approach, the use of various 3D models is more valuable – e.g., apical out lung organoids are highly suitable for high content screening (HCS) of therapeutic options, air-liquid interphase (ALI) models for e.g., simple incorporation of immune cells, evaluation of epithelial integrity, or mucociliary clearance.

2.1. Lung organoids

Organoids are 3D cell aggregates produced *in vitro* that correspond to differentiated tissues or even ´mini-organs´. Organoids originate in tissues that contain stem cells, allowing them to self-assemble *in vitro*. The organoid field owes its progress in particular to the development of stem cell technologies over the past two decades. They can be generated from adult tissue-specific stem cells or from iPSCs (induced, pluripotent stem cells) by adding specific growth factors. The organoids spontaneously organize into organ- or tissue-like structures and the cells typical for the tissue/organ are also contained in the organoid – thus organoids are heterogeneous in respect to their cell composition in contrast to the often homogeneous feeder layer cultures. Thanks to technological advances, adult stem cells can now be cultivated over a longer period of time, e.g. adult stem cell organoids from lung (Figure 1), small intestine, liver, skin and other epithelia. The organoid structure largely depends on the ability of the respective cells for self-organization, and the size of organoids is usually very limited due to limited diffusion of nutrients and oxygen in living tissue using conventional scaffold-based techniques. Under such conditions, cells in the deeper regions of the organoid, die due to hypoxia and nutrient deprivation. It is therefore not possible with these technologies to

reproduce the anatomy of larger tissues or organs, including vascular networks. In contrast, novel technologies using a vertical, rotating incubator equipped with bioreactors allow generation of large and living organoids. CelVivo - stress free 3D (ClinoStar, Denmark) is a specific incubator, in which organoids can be cultured without addition of any scaffold like Geltrex™, VitroGel™, GrowDex™ or similar. The incubator is based on bioreactors equipped with hydration balls. This area is connected to the actual reactor by a membrane, so gas exchange takes place at all times. The single cells are transferred to the reactor in 10 ml medium and placed in the incubator. There is space for up to six bioreactors in the incubator and each reactor can be controlled independently. The bioreactors are in constant rotation, whereby one can adjust the speed and direction individually. Due to the constant movement, the single cells combine to form organoids. A camera is installed in the incubator for each position, so that the condition of the organoids can be constantly monitored until they are large enough, without having to open the incubator (CelVivo; Wrzesinski et al 2021). Not only missing vascularization, but the incorporation of immune cells into organoids poses a problem, not solved yet and needing intense research. Nevertheless, organoids are in particular useful for high content/high throughput testing of potential novel drugs and vaccines within a human organ-like structure. Generation of lung organoids and re-polarization into apical-out lung organoids used for infection with SARS-CoV-2 variants (wildtype and variants of concern (VOCs)) were recently illustrated in more detail in Posch et al (2021a, c).

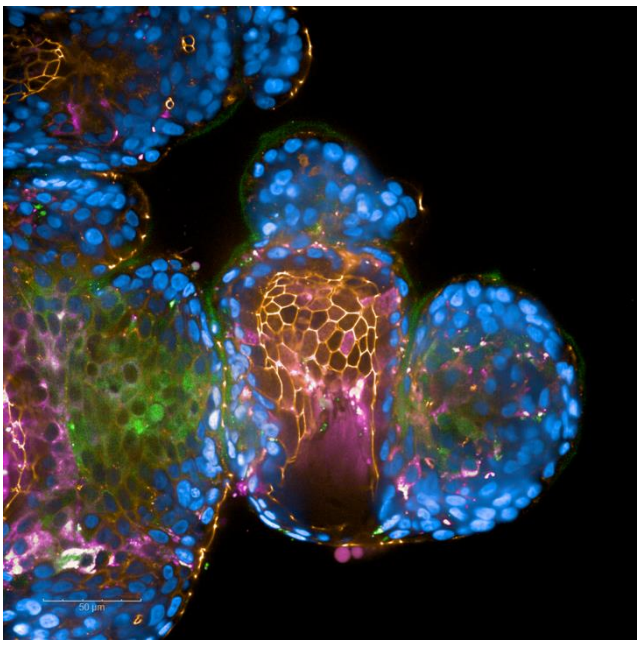


Figure 1. Lung organoids express ACE2 and TMPRSS2. Lung organoids grown in Geltrex™ were stained using the SARS-CoV-2 entry molecules ACE2 (pink) and TMPRSS2 (green), F-actin (phalloidin, orange) and nuclei (Höchst, blue).

2.2. Respiratory air-liquid interphase (ALI) cultures

In vitro approaches to recapitulate human respiratory diseases involve the use of normal human primary epithelial cells of nasal, bronchial, or tracheal origin, typically cultured on biocompatible matrices (cellulose, collagen, alginate, gelatine, elastin, Matrigel®) to mimic the *in vivo* environment. As a further step to improve the physiological relevance of these models, these primary cells are cultured under ALI. Under these conditions, the cells differentiate into a stratified (pseudostratified) epithelium containing basal cells, ciliated cells, and mucus-producing goblet cells. Complex 3D *in vitro* systems, which contain immune cells in addition to the airway epithelia mentioned above, and are stimulated with airborne particles, are valuable tools for characterizing host-pathogen interactions in tissues of the respiratory tract. Various approaches to design

sophisticated *in vitro* systems are currently being developed, but often these lack the immune component. This can be circumvented by designing epithelial/immune cell co-cultures, where immune cells are added to differentiated barrier models of the upper and lower respiratory tract. Such immune/barrier models are particularly well suited to investigate interactions with pathogens or harmful challenges on the respiratory tract, such as SARS-CoV-2, which causes COVID-19, or bacteria, fungi and cigarette smoke. Over the last decade, a respiratory epithelial/immune model was optimized in our lab in terms of applying perfusion or to allow for repeated imaging of the same sample (Zaderer et al 2019). Perfusion was described in detail in Chandorkar et al (2017), introducing relevant immune cells for analysing epithelial/immune interactions with fungi (Chandorkar et al 2017; Luvanda et al 2021a; Luvanda et al 2021b). The work by Zaderer and al. (2019) is described in more detail herein: Live cell imaging is a very important tool to characterize cellular processes, such as proliferation and differentiation. In terms of ciliated epithelia, live cell analyses are also applied for assessing mucociliary clearance that provides a first defence against pathogens attaching to the mucous layer - this mechanism is necessary for full function of the lungs (Chateau et al 2018; Gamm et al 2017; Puchelle et al 2006; Thompson et al 1995). Various protecting molecules ensure aggregation, trapping, and killing of microbes (Whitsett et al 2015). Via one-directional cilia beating, extracellular fluid is shifted towards upper parts of the mammalian airway, and the lungs are cleared from inhaled pathogens. To allow live cell imaging and monitoring the same Transwell over time for examining differentiation of cells as well as mucociliary clearance, we switched the ´world´ of the cells and seeded the cells upside-down. This technique allows to transfer the Transwell from the original plastic well plate into a liquid drop within a glass-bottom plate under sterile conditions. The same Transwell can then be analyzed for its differentiation using live cell immunofluorescence and compounds appropriate for the live cell detection of e.g., cilia (wheat germ agglutinin), mitochondrial activity (MitoTracker, MitoSOX), nuclei (Höchst), cytoskeleton (CellMask, BacMam 2.0) as well as its mucociliary clearance capacity using fluorescently labelled beads. By this modified protocol, we found that upside-down seeding of cells within a xeno-free, birch-based cellulose hydrogel (GrowDex™, UPB Biochemicals) exerted positive effects on proliferation and differentiation of primary respiratory epithelial cells. The animal-free cellulose hydrogel comprised a significantly faster differentiation of upper and lower respiratory epithelial cells even under static conditions and cells were fully differentiated after 2 weeks compared to 3 weeks in rat-tail collagen. Moreover, upside-down seeding within cellulose enabled using the same Transwell inserts over time. In addition, mucociliary clearance can be analyzed in a more realistic setting, using the upside-down seeded cells since the cells are not constricted by plastic barriers compared to seeding cells the normal way, where cells are restrained with plastics from the Transwell chamber. Also in upside-down conditions, easy addition of immune cells is feasible due to pipetting immune cells into the upper chamber of the insert, while the air side is in the lower chamber. These optimization procedures make the upside-down well a valuable tool for repeated exposure experiments, for live cell imaging over a prolonged time as well as for monitoring and evaluating mucociliary clearance after infection or treatment with e.g., antiviral sprays (Posch et al 2021b). Thus, the ALI cultures are providing more physiologic conditions compared to organoid cultures, but are limited in high content

testing. Accordingly, the work flow in our lab is to test many antifungal or antiviral compounds in high throughput in apical-out organoids prior to studying the most promising ones in immune-competent ALI cultures. These can be equipped with more than one immune component by e.g., simultaneous addition of autologous macrophages, dendritic cells, NK cells, granulocytes, T and B cells and humoral compounds i.e., complement, within one sample, consequently more realistically reflecting the situation in the human body.

2.3. Respiratory 3D models and SARS-CoV-2

Early events, right after transmission of SARS-CoV-2 to respiratory tract tissues, determine the course of infection. In some COVID-19 patients an excessive immune response is accompanied with a hyperinflammatory milieu resulting in cytokine storm and acute respiratory distress syndrome (ARDS). These are associated with increased morbidity and mortality, tissue-injuries and multi-organ failure (Chen et al 2020; Huang et al 2020; Magro 2020; Tang et al 2020; Wang et al 2020; Zhu et al 2020). To evaluate the very first interactions of SARS-CoV-2 patient isolates with human epithelial tissues, 3D models of the human respiratory tract as well as lung organoids are highly suitable.

By using our established models, we were able to

(i) detect that SARS-CoV-2 mediates mucus hypersecretion and mucus plug formation in respiratory tissues, which was also illustrated in seriously ill COVID-19 patients with airway obstruction and respiratory failure (Khan et al 2021; Posch et al 2021c);

(ii) uncover mechanisms of local complement hyperactivation upon SARS-CoV-2 infection of apical-out lung organoids as well as pseudostratified human airway epithelial cells at an air-liquid interphase. The local complement production aggravated coronavirus infection by triggering release of pro-inflammatory cytokines e.g., IL-1β, IL-6, RANTES, MCP-1 from non-immune epithelial barriers. By blocking C5aR at the basolateral side of the barrier, all these effects were reverted, tissue integrity was remained and virus infection significantly decreased (Posch et al 2021a).

(iii) identify an antiviral spray (ColdZyme™, Enzymatica) that entirely blocked binding of SARS-CoV-2 as well as local complement C3 production and associated with that, reduced infection and destruction of the tissue model. Our *in vitro* data suggest that ColdZyme mouth spray has an impact on the prevention of COVID-19 and that it is important to test the effectiveness of already approved antiviral drugs to check their effectiveness against SARS-CoV-2 (Posch et al 2021b)

These are only some examples, where various 3D tissue model systems provide valuable information on infection processes or novel treatment options, which can be expanded infinitely. Human 3D cell culture systems are suitable not just for studying first host-pathogen interactions and virus dynamics, but also offer added value especially in the preclinical phase for testing the effects of new and innovative therapeutic or repurposed interventions.

Abbreviations

ALI	Air-liquid interphase	IL	Interleukin
C3	Complement component C3	iPSC	Induced pluripotent stemcell
HCS	High content screening	VOC	Variant of concern

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