Biofabricating Airway Epithelial Models for COVID-19 Research

Himjyot Jaiswal, PhD, Isabella Bondesson, MSc, Volodymyr Kuzmenko, PhD,
Elin Pernevik, MSc, and Itedale Namro Redwan, PhD
CELLINK AB
Arvid Wallgrens Backe 20
43146, Gothenburg, Sweden

Abstract

Predictive *in vitro* cell-based models can potentially support the international effort to develop new vaccines and other treatments for lung-related diseases such as COVID-19, chronic obstructive pulmonary disease or idiopathic pulmonary fibrosis. Combining 3D biofabrication with air liquid interface culturing enables the engineering of tissue models that recapitulate typical features of the respiratory tract *in vitro* in both healthy and diseased states. These models will not only present opportunities to deeply understand the underlying mechanisms of the viral interactions with host cells at target sites but will also help reduce the number of animals used in future studies, hence supporting the 3Rs (Replacement, Reduction and Refinement) principle. In this study, we describe the generation of a 3D biofabricated airway epithelial model and evaluation of its physiological relevance. The model is characterized by the expression of angiotensin-converting enzyme 2 (ACE2), a protein required for the internalization of the coronavirus. The localization of the ACE2 protein at the apical membrane shows that the epithelial cells are polarized, and the presence of the mucin 5AC protein indicates that the model can produce the airway surface liquid, a physiological function of airway epithelial cells. Thus, these bioengineered tissue models can be used for the development of different therapeutics and vaccines.

Introduction

The novel coronavirus disease (COVID-19) that emerged at the end of 2019 is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The virus is highly transmittable and uses angiotensin-converting enzyme 2 (ACE2) as a main receptor to enter mammalian cells. ACE2 is a cell surface protein present in many cells and tissues, including the lungs, heart, blood vessels, kidneys, liver and gastrointestinal tract (Hamming, 2004). ACE2 is moderately expressed in healthy lung tissue and highly expressed in pathological conditions, like chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), asthma, diabetes and hypertension (Saheb Sharif-Askari, 2020). The SARS-CoV-2 is known to infect the respiratory tract and spread throughout the human body, leading to multiple organ infection symptoms (de Melo, 2021). Therefore, due to the severe effects of the virus on human health, the development of new diagnostic methods, vaccines and antiviral drugs is urgent.

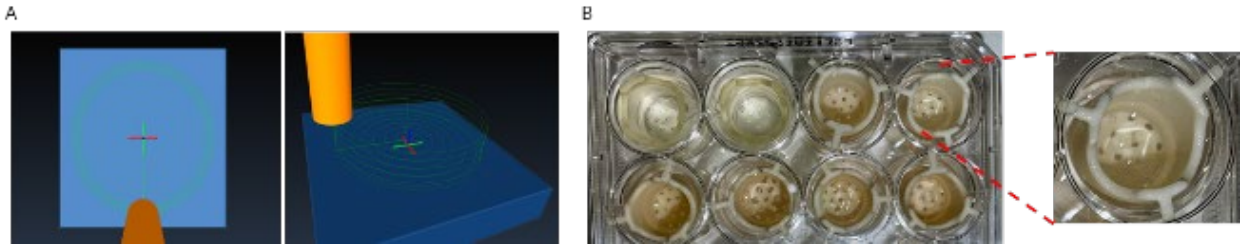

Figure 1. ***(A)*** *Illustration of the 3D bioprinted model using CAMotics, top view (left) and side view (right).* ***(B)*** *3D bioprinted lung tissue models transferred to Transwell inserts.*

In vitro models are crucial in understanding disease mechanisms as well as in validating therapeutics before clinical trials. Although current 2D culture models are common for screening virus replication, for infection and drug screening, they fail to recapitulate the complexity and physiology of the tissue. New platforms, like 3D bioprinting technology, however, can generate tissue models that better resemble the *in situ* tissue in

terms of their molecular composition, biomechanics and complexity (Singh, 2020; Seyfoori, 2021). These 3D bioprinted models allow for a better understanding of the underlying host-pathogen interaction in a micro-environment similar to that of a human organ and thus facilitate screening of drugs against pulmonary diseases such as COVID-19.

In this study, we focused on bioprinting primary human bronchial epithelial or Calu-3 cells to generate an air liquid interface (ALI) model. Calu-3 is a well-differentiated and -characterized cell line derived from human bronchial submucosal glands (Zhu, 2010). The submucosal glands in human lungs are a major source of airway surface liquid, mucins and other immunologically active substances.

The bioprinted lung disease models were cultured in Transwell inserts for 14 days and immunostained for ACE2 and mucin 5AC (MUC5AC) proteins. Using the experimental setup presented in this application note enables the study of host-pathogen interaction with regards to SARS-CoV-2 and airway epithelial cells (Zhu, 2010).

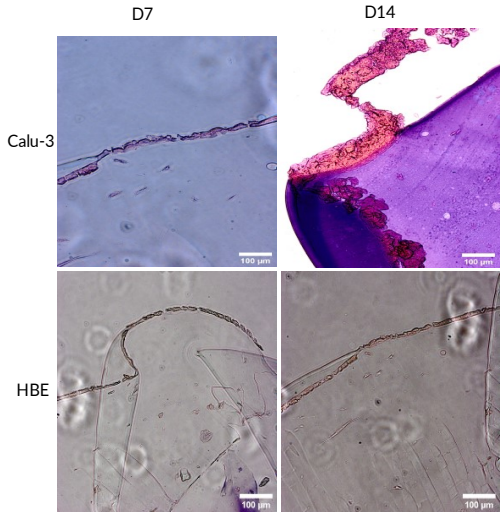

Figure 2. *H&E staining of the lung tissue model at day 7 (D7) and day 14 (D14) at 10x magnification. The epithelial layer of Calu-3 and HBE cells located on top of the construct. Scale bar = 100 µm.*

Materials and methods

Cell preparation

Two different epithelial cell types were used in the lung tissue model: human bronchial epithelial cells (HBEpC; C-12640, PromoCell) and Calu-3 (lung adenocarcinoma-derived epithelial cells; HBT-55, ATCC). The human pulmonary fibroblasts (HPF; C-12360, PromoCell) were used as supporting cells. The cells were thawed and expanded in 2D prior to being bioprinted according to the respective manufacturer's protocol. HBEpC were cultured in airway epithelial cell growth medium (C-21060, PromoCell), HPF were cultured in fibroblast growth medium 2 supplemented with growth medium 2 kit (C-23020, PromoCell) and used in passage 4 and passage 9, respectively; whereas Calu-3 cells were cultured in minimum essential medium (MEM; 51200038, Gibco Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; 10270106, Gibco Thermo Fisher Scientific) and 1% penicillin-streptomycin-neomycin mix (PSN; 15640055, Gibco Thermo Fisher Scientific) and used in passage 4.

Bioprinting of lung tissue model

The lung model was designed as a disk, with a solid bottom layer topped with a 5-layered brim (Figures 1A, 1B). This was performed to facilitate entrapment of post-seeded epithelial cells. The disk was 3D printed with GelMA-Laminin 521 (GelMA-LN521, CELLINK). Next, 2 x 10^6 HPF were trypsinized, washed, collected, spun and gently resuspended in 100 µL of fibroblast medium 2, which was then carefully mixed into 1 mL GelMA-LN521 bioink, prewarmed to 37°C and transferred to an amber cartridge (CSO010311502, CELLINK). To remove entrapped air bubbles, the cartridge of GelMA-LN521 with embedded HPFs was centrifuged at 460*g* for 1 minute. The cartridge was mounted in the Temperature-controlled Printhead (00000020346, CELLINK) set at 27°C for 20 minutes in the BIO X™ bioprinter before starting the print session. The cartridge was turned upside down two to three times to avoid sedimentation of cells. The suitable bioprinting temperature for GelMA-LN521 bioinks is 26°C to 27°C.

The disk baskets were then bioprinted at 27°C with the Temperature-controlled Printhead in a 12-well plate. Twelve disk replicates were first photocured for 10 seconds per construct using the 405 nm photocuring module (distance was set to 5 cm from the construct), and fibroblast growth medium was added to each well. The disks were then incubated for 3 days in fibroblast growth medium at 37°C prior to the addition of the epithelial layer.

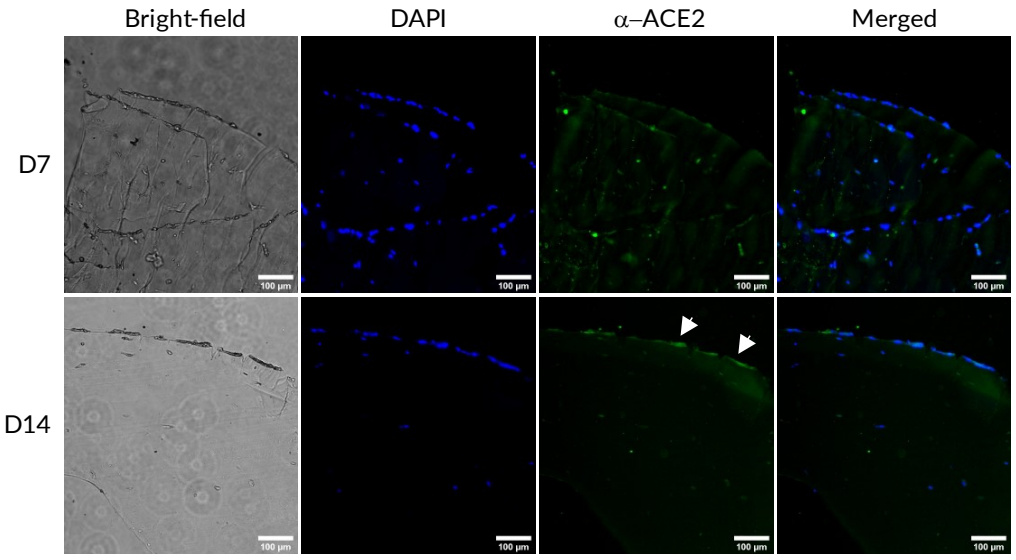

Figure 3. *Immunostaining for ACE2 in HBE cells. ACE2 (green) and nuclei stain DAPI (blue) at different days on ALI cultured constructs. Scale bar = 100 µm.*

Addition of lung epithelial layer

For the epithelial layer, HBE or Calu-3 cells were resuspended in airway epithelial growth medium or MEM complete medium, respectively, and supplemented with 5 ug/mL LN521. 150K cells/cm^2 were seeded on top of each disk. Fibroblast medium was removed from the wells, 5 x 10^5 cells were resuspended in 360 µL of complete medium, and 30 µL of cell suspension mix were added on top of each disk. The constructs were left on the benchtop for 10 minutes after the seeding and then transferred to the incubator for another 20 minutes to settle and for cells to attach to the bioprinted disk. HBE constructs were submerged gently into a mix of fibroblast and airway epithelial medium in a 1:9 ratio; and Calu-3 constructs were submerged into a mix of fibroblast and MEM medium in a 1:9 ratio. The submerged culture was grown for 4 days at 37°C, 5% CO_2, before being transferred to ALI culture. ALI culture was established by transferring the constructs into Transwell inserts supplemented with a suitable volume of identical medium as described above (Figure 1B). Medium was refreshed every 2nd to 3rd day.

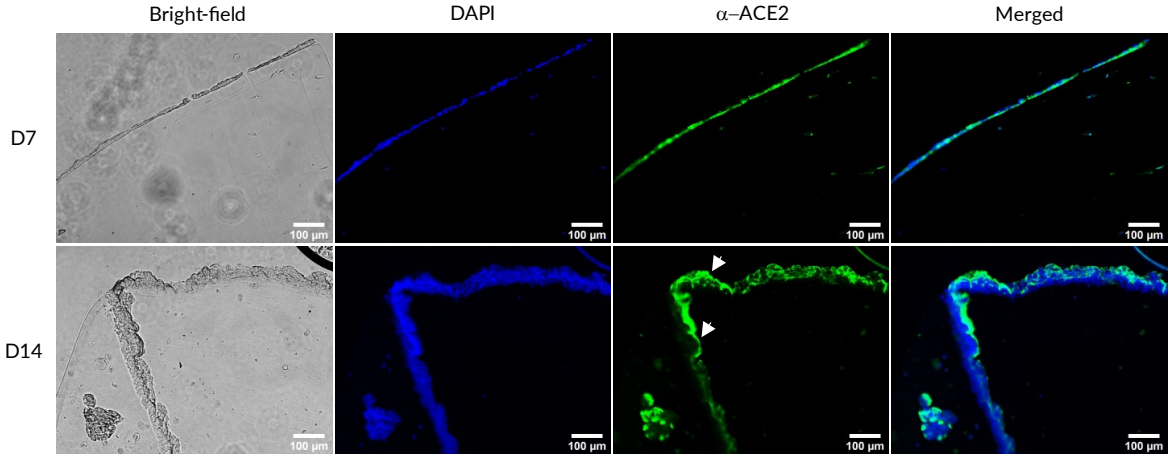

Figure 4. *Immunostaining for ACE2 in Calu-3 cells. Cells were grown on GelMA-LN521 in an ALI culture. At day 7 (D7) and day 14 (D14), cells were fixed, sectioned and stained for ACE2 (green). Nuclei were visualized using DAPI (blue). The constructs were visualized in bright-field to see the ALI. Scale bar = 100 µm.*

Analysis

Samples were collected at day 7 and day 14 (ALI culture) and fixed for histology staining in 4% para-formaldehyde (PFA), according to CELLINK's fixation protocol. The samples were then embedded in paraffin, and 10 µm sections were obtained using a microtome, following CELLINK's sectioning protocol. The sections were stained for ACE2 (MAB933, R&D Systems) and MUC5AC (AB3649, Abcam), following CELLINK's immunofluorescence protocol and using Alexa Fluor 488 (A-11029, Thermo Fisher Scientific) as a secondary antibody. The samples were also stained with H&E staining, following CELLINK's protocol. All protocols can be found on CELLINK's website, under the Support tab.

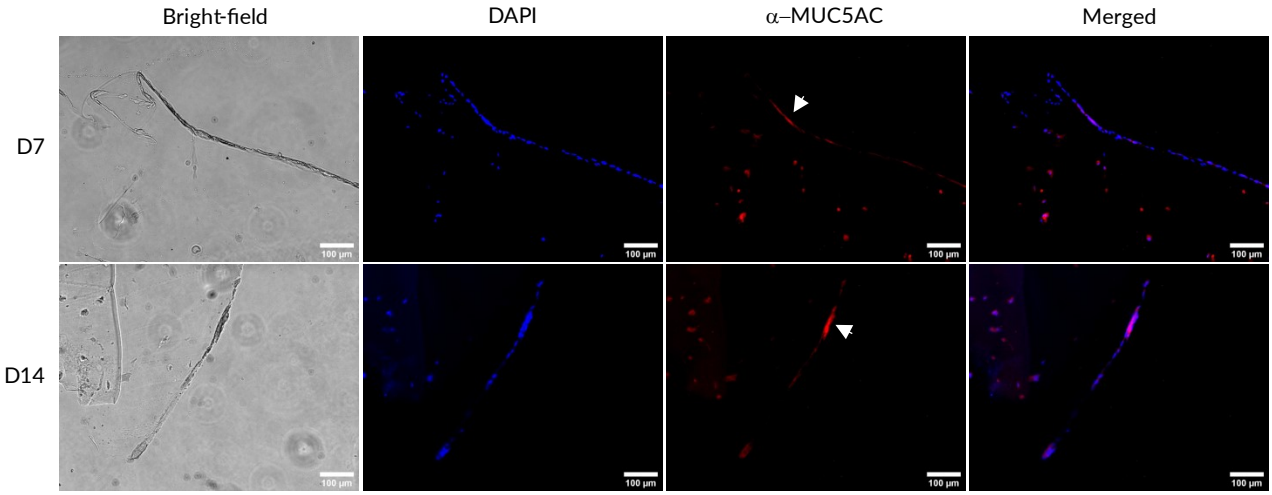

Figure 5. Immunostaining for mucin in HBE cells. Cells were grown on GelMA-LN521 in an ALI culture. At day 7 (D7) and day 14 (D14), cells were fixed, sectioned and stained for MUC5AC (red). Nuclei were visualized using DAPI (blue). The constructs were visualized in bright-field to see the ALI. Scale bar = 100 µm.

Results and discussion

The fabrication method used for generating the lung tissue model created an intact and robust construct that maintained its shape throughout the experiment. The H&E staining of the sample cross-sections showed that both Calu-3 and HBE cells seeded on top of the construct formed a tight monolayer at day 7. However, at day 14, the Calu-3 cells formed a thick multilayered epithelial-like structure, whereas the HBE cells still maintained a thin monolayer (Figure 2). This indicates that the primary bronchial epithelial cells maintained contact inhibition, which was lost in cancer cells, leading to cells growing on top of each other.

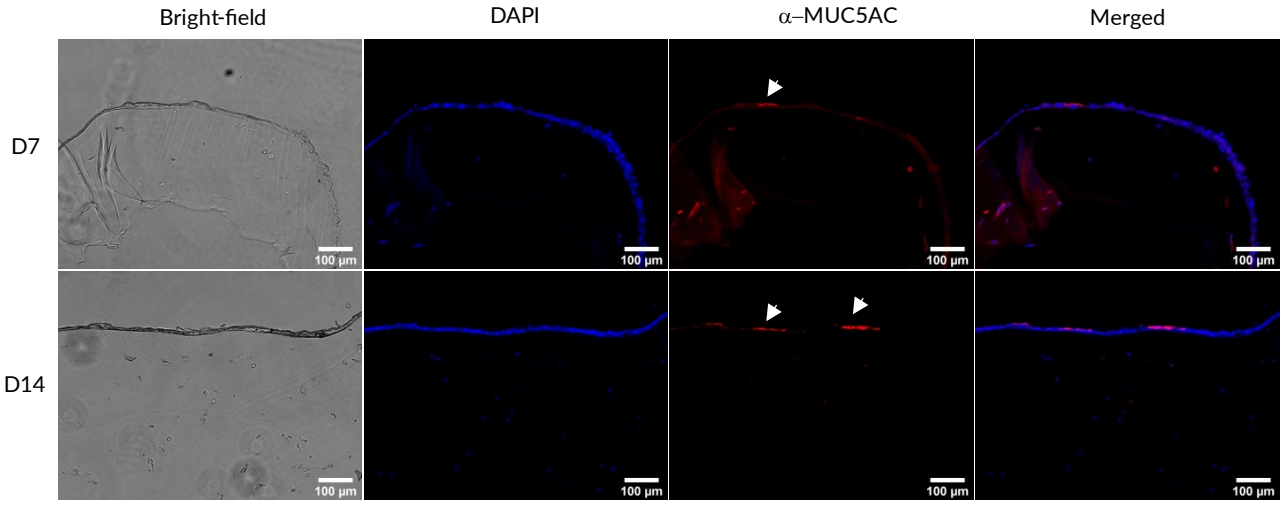

Figure 6. Immunostaining for mucin in Calu-3 cells. Cells were grown on GelMA-LN521 in an ALI culture. At day 7 (D7) and day 14 (D14), cells were fixed, sectioned and stained for MUC5AC (red). Nuclei were visualized using DAPI (blue). The constructs were visualized in bright-field to see the ALI. Scale bar = 100 µm.

Next, we performed immunostaining for ACE2, a cell surface protein involved in the internalization of the coronavirus. In the HBE samples, very few cells stained for ACE2 in the monolayer at day 7, whereas at day 14 the ACE2 stain was detected in many cells (Figure 3). Contrary to HBE, most of the cells in the Calu-3 sample stained for ACE2 both at day 7 and day 14 (Figure 4). Also, immunostaining for ACE2 was observed at the apical membrane domain (air surface in ALI culture) as seen by the localized green signal toward the top Calu-3 cells in day 14 samples (Figure 4), indicating that the cell layer is polarized. Together these data suggest that our Transwell model shows the polarized airway epithelial cells with expression of ACE2 in both primary and cancer-derived cells.

To further characterize the model, we performed immunostaining for mucin in ALI cultured constructs as MUC5AC has been reported to be produced by goblet cells in tracheobronchial surface epithelium (Kesimer, 2009). We observed the expression of MUC5AC in HBE and Calu-3 cells in the ALI cultured models (Figures 5, 6), indicating that the cells had differentiated. Together our data suggests that the 3D biofabricated model presented here will provide an excellent tool to engineer 3D lung tissue models for the rapid screening of biologics against COVID-19. This will also facilitate the reduction of animal studies, thus supporting the 3Rs principle.

Conclusion

This study demonstrates that 3D bioprinting combined with Transwell cell culturing could be used to generate complex *in vitro* lung epithelial models.

- The *in vitro* ALI model showed expression of ACE2 and MUC5AC proteins.
- The primary bronchial epithelial cells maintained the tight monolayer throughout the experiment, whereas the Calu-3 cells formed multilayer epithelium at day 14.
- The expression of ACE2 was moderate in primary bronchial epithelial cells compared to adenocarcinoma-derived Calu-3 cell line.
- The cells grown in ALI culture became polarized as seen by the expression of ACE2 at the apical membrane of the culture.

References

1. Hamming I, Timens W, Bulthuis MLC, Lely AT, Navis GJ, van Goor H. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *The Journal of Pathology*. 2004; 203(2): 631–637. DOI:10.1002/path.1570.
2. Saheb Sharif-Askari N, Saheb Sharif-Askari F, Alabed M. Airways expression of SARS-CoV-2 receptor, ACE2, and TMPRSS2 is lower in children than adults and increases with smoking and COPD. *Molecular Therapy - Methods & Clinical Development*. 2020; 18: 1–6. DOI:10.1016/j.omtm.2020.05.013.
3. de Melo BAG, Benincasa JC, Cruz EM, Maricato JT, Porcionatto MA. 3D culture models to study SARS-CoV-2 infectivity and antiviral candidates: From spheroids to bioprinting. *Biomedical Journal*. 2021; 44(1): 31–42. DOI:10.1016/j.bj.2020.11.009.
4. Singh AK, Mishra G, Maurya A, Kulkarni GT, Awasthi R. Biofabrication: An interesting tool to create in vitro model for COVID-19 drug targets. *Medical Hypotheses*. 2020; 144: 110059. DOI:10.1016/j.mehy.2020.110059.
5. Seyfoori A, Amereh M, Dabiri SMH, Askari E, Walsh T, Akbari M. The role of biomaterials and three dimensional (3D) in vitro tissue models in fighting against COVID-19. *Biomaterials Science*. 2021; 9(4): 1217–1226. DOI:10.1039/D0BM01616K.
6. Zhu Y, Chideke IA, Shaffer TH. Cultured human airway epithelial cells (Calu-3): A model of human respiratory function, structure, and inflammatory responses. *Critical Care Research and Practice*. 2010: 394578. DOI:10.1155/2010/394578.
7. Kesimer M, Kirkham S, Pickles RJ. Tracheobronchial air-liquid interface cell culture: a model for innate mucosal defense of the upper airways? *American Journal of Physiology-Lung Cellular and Molecular Physiology*. 2009; 296(1): L92–L100. DOI:10.1152/ajplung.90388.2008.

Contact us

U.S. phone:
(+1) 833-235-5465

Email:
sales@cellink.com

European phone:
+46 31-128 700

Website:
www.cellink.com