

3D Airway Models for In Vitro Studies and Drug Delivery

Aurélie Marches^{1,2}, Sara Gouarderes³, Anna-Karin Larsson-Callerfelt⁴ & Sabrina Valetti^{1,2}

¹Department of Biomedical Sciences, Faculty of Health and Society, Malmö University, 205 06 Malmö, Sweden

²Biofilms Research Center for Biointerfaces (BRCB), 205 06 Malmö, Sweden

³RESTORE, 31100 Toulouse, France

⁴Lung Biology, Department of Experimental Medical Science, Lund University, 22184 Lund, Sweden

Summary

Aligned with the 3R principles and the need for physiologically relevant alternatives to animal testing, this study aims to develop advanced 3D human bronchial airway models using a scaffold-free cell sheet engineering approach. Unlike standard air liquid interface (ALI) monocultures, our model includes stromal components and native-like extracellular matrix (ECM) produced by ascorbic acid-stimulated lung fibroblasts. This technique preserves critical cell-cell and cell-ECM interactions, avoids scaffold-related drawbacks, and offers cost-efficiency and high reproducibility. The full-thickness model was established with BCI-NS1.1 bronchial basal epithelial cells resting on an HFL1 fibroblast-derived connective tissue layer that better replicates the *in vivo* airway microenvironment. A split-thickness model (i.e., only epithelial cells) on permeable inserts was established as comparison. Both models were cultured at the air-liquid interface for up to 28 days. Histology and immunofluorescence confirmed pseudostratified epithelia composed of basal (KRT14+), ciliated (FOXJ1+), and mucus-producing goblet cells (MUC5AC+). Trans-epithelial electrical resistance measurements indicated functional barrier formation. The full-thickness model demonstrated similar epithelial organization to the split-thickness model but showed reduced goblet cell differentiation, potentially due to ECM-related effects on epithelial maturation. Importantly, the full-thickness model showed cellular proportions comparable to native airway tissue and published models, supporting its relevance as an advanced *in vitro* model for pharmaceutical studies. This study introduces a robust, human-relevant airway tissue model with integrated stroma, providing a valuable platform for disease modelling and inhalation toxicology.

Key Message

This study presents a scaffold-free, full-thickness 3D bronchial model using BCI-NS1.1 epithelial cells atop fibroblast-derived ECM, and a split-thickness model, with only epithelial cells on permeable inserts. Both produced a differentiated pseudostratified epithelium with ciliated and mucus-producing goblet cells and showed promise as advanced *in vitro* models for pharmaceutical studies.

Introduction

In line with the 3R principles (Replacement, Reduction, and Refinement) that promote alternatives to animal use in research, tissue engineering has emerged as a promising strategy for reconstructing human lung mucosa for various applications in lung research and testing [1], [2]. However, current *in vitro* and *ex vivo* models still fall short of replicating the complex cellular architecture and physiological microenvironment of the human airway, particularly in terms of cell-cell, extracellular matrix (ECM)-cell, and biomechanical interactions. The prevailing standard model, monocultures of human bronchial epithelial cells grown at the air-liquid interface (ALI), supports epithelial differentiation and mimics key functions such as mucus production, cilia, and barrier properties [2][3]. While this model has significantly contributed to our understanding of airway biology, disease mechanisms, and therapeutic responses, it remains limited by the absence of non-epithelial cell types, minimal ECM interactions and the use of rigid culture substrates that do not reflect the native tissue mechanics. These non-physiological conditions influence epithelial cell phenotype and restrict the model's ability to accurately simulate complex airway diseases that involve the surrounding tissue environment [4][5]. Therefore, there is a benefit from advanced, full-thickness 3D *in vitro* airway models that integrate a well-differentiated human airway epithelium at ALI with physiologically relevant interactions with the underlying connective tissue. Such models would offer improved platforms for studying normal and diseased airway biology, drug screening, and pulmonary delivery research.

We have used a scaffold-free tissue engineering approach based on a cell sheet engineering technique, also referred to as the self-assembly method [6]. This method preserves native ECM and cell-cell interactions, while eliminating the need for synthetic or natural scaffolds, avoiding potential issues such as immunogenicity, toxicity, or interference with cell behavior and tissue remodeling. Key advantages also include lower material and logistical costs compared to scaffold-based or *in vivo* models, high reproducibility, the ability to incorporate multiple cell types, and the potential for genetic modification [6]. In this strategy, cells are cultured for approximately four weeks in a medium supplemented with ascorbic acid, which stimulates them to secrete and organize their own ECM, effectively creating a native-like

tissue structure without the need for exogenous synthetic or animal-derived biomaterials. This technique enables the generation of fully autologous, three-dimensional tissue constructs with enhanced physiological relevance. The self-assembly method has been successfully applied to engineer a variety of human tissues, including skin, blood vessels, cornea, and adipose tissue, for both research and clinical purposes [7]. However, it has yet to be adapted or optimized for the development of pulmonary tissue models.

The objective of this study was to develop layered tissue-engineered models of the human bronchial airway that closely mimic the characteristics of *in vivo* tissue, that are structurally robust, and user-friendly, aiming to support both mechanistic studies of lung biology and the development of inhalation therapies. The project was structured around two main work packages: firstly, the optimization of split-thickness bronchial epithelium models, focusing on establishing a fully differentiated muco-ciliated epithelium grown at the air–liquid interface. Secondly, a full-thickness bronchial model, consisting of a well-differentiated bronchial epithelium cultured on top of a connective airway tissue layer composed of fibroblasts embedded within their self-produced extracellular matrix.

Methods

Cell Culture BCI-NS1.1 bronchial cell line was obtained from the laboratory of Dr. Ronald Crystal, Cornell University (NY, United States of America) [8]. BCI-NS1.1 cells were utilized to produce the split-thickness models of bronchial epithelium models. Cell culture was performed as reported by [8]. Briefly, cells were seeded on Sarstedt cell inserts, polyethylene terephthalate (PET) membrane (growth area 1.1 cm², pore size 0.4 µm), coated with 1% Collagen type I, Rat Tail (Merck), air-lifted after 2 days and allowed to differentiate at ALI for 21 and 28 days.

Tissue engineering Lung fibroblasts HFL1 were used to produce reconstructed lung connective tissue, with an adapted protocol previously shown by Gibot et al., [9] for reconstructed dermis with skin fibroblasts. Briefly, HFL1 were grown for 21 days with 50 mM ascorbic acid, before BCI-NS1.1 cells were seeded on the apical side, cultivated 5 days in submerged culture before lifting at ALI for an additional 21 days.

TEER Trans-epithelial electrical resistance at 12.5 Hz was measured using Sensit/BT potentiostat (PalmSens, Netherlands).

Histology After 21 or 28 day at ALI, samples were fixated and embedded in paraffin. Haematoxylin Eosin (HE), Periodic Acid Schiff (PAS) and Alcian Blue were performed according to standard procedures.

Immunofluorescence After 21 or 28 day at ALI, samples were fixated and embedded in paraffin, immunostaining of cross-sections with an adapted protocol from [10], was performed using antibodies targeting markers associated with three of the major cell types of the airways [8], [11]: Keratin 14 (KRT14), basal cells (mouse, monoclonal, IgG, Abcam), MUC5AC, goblet cells (mouse, monoclonal, IgG, Abcam), FOXJ1, ciliated cells, (mouse, monoclonal, IgG, Invitrogen). Secondary antibody was Alexa 647-conjugated anti-mouse (goat, polyclonal, IgG, Invitrogen).

Results and discussion

In this study, *in vitro* 3D lung equivalents were developed as split- and full- thickness models of bronchial airway.

Split-thickness model: BCI-NS1.1 cells were cultured on permeable membranes at the air-liquid interface (ALI) for 21 or 28 days. Although standard protocols recommend 28 days of ALI culture [8], shorter durations were also tested to optimize differentiation.

Histological analysis showed a pseudostratified epithelium containing ciliated and goblet cells (Figure 1A). Immunofluorescence for the basal cell marker KRT14 (Figure 1B) indicated that many basal cells remained undifferentiated at both 21 and 28 days.

Goblet cell differentiation was confirmed using by PAS-Alcian Blue and MUC5AC staining (Figures 1A-B). Quantification showed an increase in Alcian Blue-positive secretory cells from $33.6 \pm 6.7\%$ at day 21 to $49.5 \pm 8.8\%$ at day 28, along with stronger MUC5AC signal intensity, indicating enhanced mucus production at later stages of differentiation. These results aligned with Walters et al. [8] BCI-NS1.1 cells model and Hedström et al. [11] model using normal human bronchial epithelial cells on decellularized human bronchial scaffolds.

Immunostaining for the ciliated cell marker FOXJ1 (Figures 1B-C) showed a decrease of FOXJ1-positive ciliated cells from $33 \pm 7.2\%$ at day 21 to $14 \pm 2.1\%$ at day 28. These results contrasted with the findings of Walters et al. [8] and Hedström et al. [11]. After 28 days of ALI culture, an increase in goblet cells accompanied by a reduction in ciliated cells was observed, suggesting a shift toward goblet cell differentiation over time.

Barrier integrity assessed by TEER measurements averaged $617 \pm 30.6 \Omega \cdot \text{cm}^2$ (21 days) and $515 \pm 147.6 \Omega \cdot \text{cm}^2$ (28 days), a moderate value consistent with fully differentiated airway cultures [12]. Compared to Walters *et al.* [8], which showed higher TEER values ($1312 \pm 281 \Omega \cdot \text{cm}^2$) but lower differentiation (8-15% ciliated and 1-5% goblet cells), our model displayed a greater proportion of differentiated columnar cells. These results suggest that as epithelial cells mature into ciliated and secretory phenotypes, cell packing density and tight junction tightness decrease, leading to reduced TEER values, consistent with previous Calu-3 cells studies [10], [13].

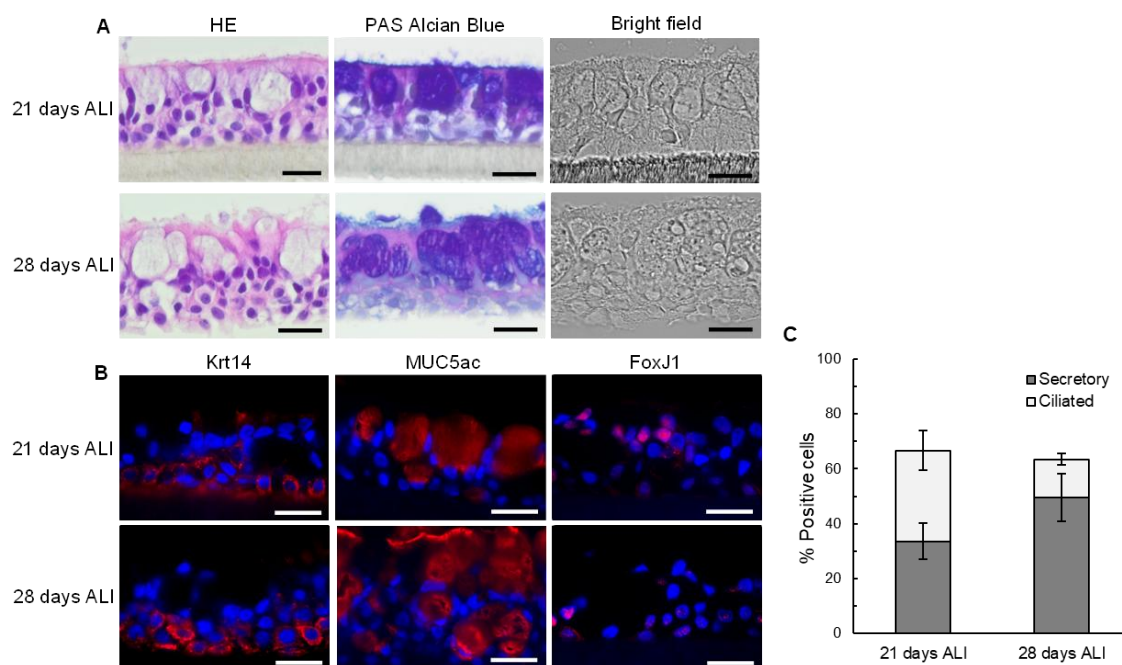


Figure 1. Morphology of bronchial epithelium split-thickness airway model at ALI day 21 and 28 with BCI-NS1.1 cells. A. Representative histological images after haematoxylin and eosin (HE) staining, Periodic Acid Schiff (PAS) / Alcian Blue staining. Representative confocal microscopy image in brightfield. Scale bars are 20 μm . B. Representative confocal microscopy images of immunofluorescence staining with differentiation marker antibodies specific for KRT14 (red), MUC5AC (red), and FOXJ1 (red) while nucleus staining (DAPI) is in blue. Scale bars are 20 μm . C. Percentage of positive cells for alcian blue staining (secretory cells) and FoxJ1 (ciliated cells) were quantified using ImageJ. Data are presented as the mean $\pm$ S.D. (n = 3).

Full-thickness model: The full-thickness model consisted of a connective tissue compartment containing HFL1 fibroblasts, cultured for 21 days to allow them to produce and embed within their own extracellular matrix. On top of this layer, BCI-NS1.1 epithelial cells were seeded and cultured at ALI for 21 days, forming a pseudostratified epithelium.

As shown in Figure 2A, fibroblasts were well embedded in collagen fibers, forming a 40-100 μm thick connective tissue supporting the epithelial layer. Similar to the split-thickness model, the pseudostratified epithelium contained basal, ciliated, and goblet cells after 21 days at ALI. KRT14 (Figure 2B) was mainly expressed in basal cells, confirming their phenotype, though a few apical cells also showed KRT14 staining, possibly due to non-specific labeling or regeneration activity.

PAS-Alcian blue staining confirmed differentiation of BCI-NS1.1 cells into goblet cells, with $13.8 \pm 5\%$ Alcian Blue-positive secretory cells (Figures 2B-C), about half the proportion observed in the split-thickness model at the same time point, suggesting less advanced differentiation in the full-thickness model.

FOXJ1 immunostaining (Figures 2B-C) revealed $36 \pm 6.4\%$ ciliated cells, comparable to the split-thickness model ($33 \pm 7.2\%$), indicating good epithelial coverage by ciliated cells.

Overall, the epithelial organization and cell-type distribution of the epithelial compartment were similar to those reported by Hedström *et al.*[11] ($\approx 25\%$ MUC5AC⁺, 8% FOXJ1⁺ after 21 days) and were comparable to healthy human airway tissue cell type proportions [14], approximately 31% basal, 10.6% secretory, and 10.7% ciliated cells. These findings suggest that the full-thickness bronchial airway model in development in this study represents a promising in vitro model for lung research and pharmaceutical applications.

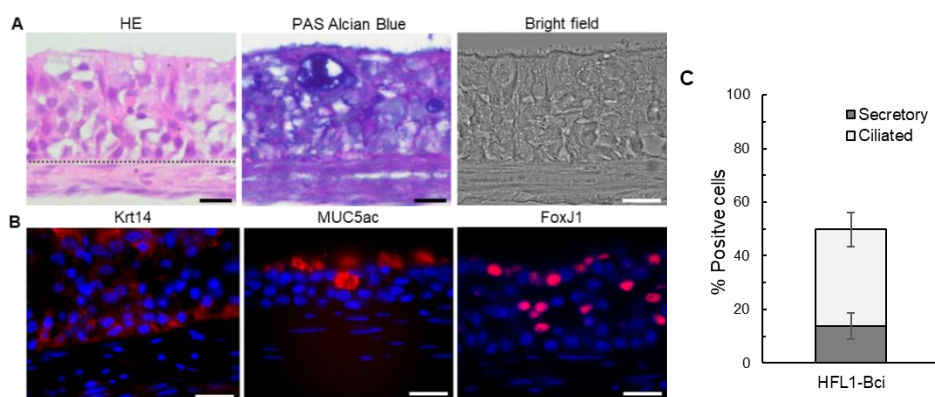


Figure 2. Morphology of the full-thickness airway model with BCI-NS1.1 bronchial cells and HFL1 lung connective tissue after 21 days at ALI. **A.** Representative histological images after hematoxylin and eosin (HE) staining, Periodic Acid Schiff (PAS) / Alcian Blue staining. The dotted line represents the delimitation between the epithelial layer and the stromal layer. Representative confocal microscopy image in brightfield. Scale bars are 20 μ m. **B.** Representative confocal microscopy images of immunofluorescence staining with differentiation marker antibodies specific for KRT14 (red), MUC5AC (red), and FOXJ1 (red) nucleus staining (DAPI) is in blue. Scale bars are 20 μ m. **C.** Percentage of positive cells for alcian blue staining (secretory cells) and FoxJ1 (ciliated cells) were quantified using ImageJ. n = 3 Error bars represent S.D.

Conclusion

In this study we developed a novel *in vitro* 3D human bronchial airway equivalent as split- and full-thickness models using BCI-NS1.1 cells. The full-thickness model featured a pseudostratified epithelium supported by a fibroblast-derived ECM-rich connective tissue. This model is comparable to healthy human airway tissue cell type proportion, with about 13.8% of secretory cells and 36% of ciliated cells.

Acknowledgments

This research was funded by the Knowledge Foundation KK-stiftelsen grant number 20190010. Oskar Hallgren (Truly Labs) is acknowledged for the discussion concerning the bronchial epithelial cells biology. Carolina Robertsson and Zita Lopez (Lund University and Malmö Odontology Faculty) are acknowledged for the training on the Spinning Disc confocal microscope.

References

- [1] J. K. Metz, M. Hittinger, and C.-M. Lehr, "In vitro tools for orally inhaled drug products—state of the art for their application in pharmaceutical research and industry and regulatory challenges," *In vitro models*, vol. 1, no. 1, pp. 29–40, Feb. 2022.
- [2] X. Cao, J.P. Coyle, R. Xiong, Y. Wang, R.H. Heflich, B. Ren, W.M. Gwinn, P. Hayden, and L. Rojanasakul, "Invited review: human air-liquid-interface organotypic airway tissue models derived from primary tracheobronchial epithelial cells—overview and perspectives," *In Vitro Cell.Dev.Biol.-Animal*, vol. 57, no. 2, pp. 104–132, Feb. 2021.
- [3] R. G. Crystal, S. H. Randell, J. F. Engelhardt, J. Voynow, and M. E. Sunday, "Airway Epithelial Cells," *Proc Am Thorac Soc*, vol. 5, no. 7, pp. 772–777, Sep. 2008.
- [4] B.-H. Gu, M. C. Madison, D. Corry, and F. Kheradmand, "Matrix remodeling in chronic lung diseases," *Matrix Biology*, vol. 73, pp. 52–63, Nov. 2018.
- [5] S. M. Busch, Z. Lorenzana, and A. L. Ryan, "Implications for Extracellular Matrix Interactions With Human Lung Basal Stem Cells in Lung Development, Disease, and Airway Modeling," *Front. Pharmacol.*, vol. 12, May 2021.
- [6] K. A. Athanasiou, R. Eswaramoorthy, P. Hadidi, and J. C. Hu, "Self-Organization and the Self-Assembling Process in Tissue Engineering," *Annu Rev Biomed Eng*, vol. 15, pp. 115–136, 2013, doi: 10.1146/annurev-bioeng-071812-152423.
- [7] M. Yamato and T. Okano, "Cell sheet engineering," *Materials Today*, vol. 7, no. 5, pp. 42–47, May 2004.
- [8] M. S. Walters, K. Gomi, B. Ashbridge, M.A.S. Moore, V. Arbelaez, J. Heldrich, B.S. Ding, S. Rafii, M.R. Staudt, and R.G. Crystal, "Generation of a human airway epithelium derived basal cell line with multipotent differentiation capacity," *Respir Res*, vol. 14, no. 1, p. 135, 2013.
- [9] L. Gibot, T. Galbraith, J. Bourland, A. Rogic, M. Skobe, and F. A. Auger, "Tissue-engineered 3D human lymphatic microvascular network for in vitro studies of lymphangiogenesis," *Nature Protocols*, vol. 12, no. 5, Art. no. 5, May 2017.
- [10] J. E. Campos Pacheco, A. Vitali, P. Falkman, A. Marauri, A. Marches, H. Al-Najjar, A. Colom, M. Cárdenas, G. A. Pilkington, S. Valetti, "Bioinspired lipid coated porous particle as inhalable carrier with pulmonary surfactant adhesion and mucus penetration," *Journal of Colloid and Interface Science*, vol. 697, p. 137967, Nov. 2025.
- [11] U. Hedström, O. Hallgren, L. Öberg, A. DeMicco, O. Vaarala, G. Westergren-Thorsson and X. Zhou, "Bronchial extracellular matrix from COPD patients induces altered gene expression in repopulated primary human bronchial epithelial cells," *Sci Rep*, vol. 8, p. 3502, Feb. 2018.
- [12] S. Chen, R. Einspanier, and J. Schoen, "Transepithelial electrical resistance (TEER): a functional parameter to monitor the quality of oviduct epithelial cells cultured on filter supports," *Histochem Cell Biol*, vol. 144, no. 5, pp. 509–515, 2015.
- [13] "TEER values: What do they tell you about your ALI cultures?" Accessed: Jul. 10, 2025. [Online]. Available: <https://www.stemcell.com/correlating-teer-values-with-air-liquid-interface-culture-morphology.html>
- [14] M. Deprez, L.E. Zaragosi, M. Truchi, C. Becavin, S. Ruiz García, M.J. Arguel, M. Plaisant, V. Magnone, K. Lebrigand, S. Abelanet, F. Brau, A. Paquet, D. Pe'er, C.H. Marquette, S. Leroy and P. Barbry, "A Single-Cell Atlas of the Human Healthy Airways," *Am J Respir Crit Care Med*, vol. 202, no. 12, pp. 1636–1645, Dec. 2020.