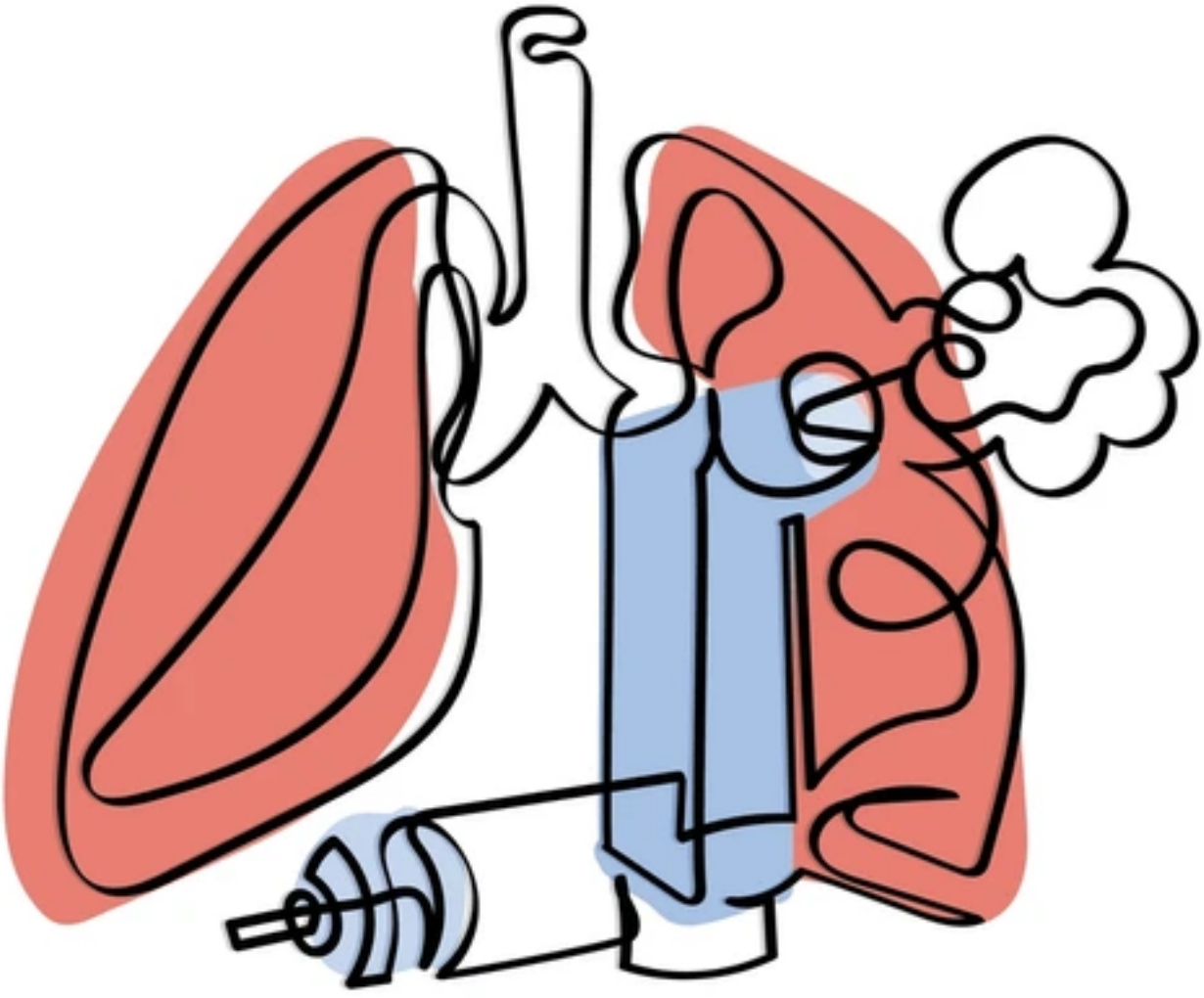
Temperature-Driven Modulation of Cellular Differentiation, Mucociliary Function, and Host Defense Responses of the Airway Epithelium

Elena Marie-Louise Ferraris



TU Delft Delft University of Technology

LUMC Leids Universitair Medisch Centrum

Note. Shutterstock contributor. (2024)

DELFT UNIVERSITY OF TECHNOLOGY

BIOMEDICAL ENGINEERING
MASTER THESIS

Temperature-Driven Modulation of Cellular Differentiation, Mucociliary Function, and Host Defense Responses of the Airway Epithelium

Author:
Elena Marie-Louise Ferraris (5657180)

In Partial Fulfilment of the Requirements of
Master of Science in Biomedical Engineering
track Medical Devices (Track II)
at the Delft University of Technology,
to be defended publicly on Monday February 17th, 2025

Thesis Committee:
Dr. Massimo Mastrangeli[1] (Chair)
Dr. Anne van der Does[2]
Dr. ir. Frank Gijsen[3]
Dr. Roberto Narcisi[4]
Lars Wessels[2], BSc.

Thesis advisors:
Dr. Massimo Mastrangeli
Dr. Anne van der Does
Lars Wessels, BSc.

[1]*Department of Microelectronics, Delft University of Technology, Delft*
[2]*Department of Pulmonology, Leiden University Medical Center, Leiden*
[3]*Department of Biomechanical Engineering, Delft University of Technology, Delft*
[4]*Department of Orthopaedics & Sports Medicine, Erasmus Medical Center, Rotterdam*

TU Delft Delft University of Technology

LUMC Leids Universitair Medisch Centrum

PREFACE

Regulation of the cellular composition and function of the airway epithelium is a critical aspect of the respiratory defense and maintenance of homeostasis. Understanding the mechanisms underlying these cellular dynamics is essential to understand the role of the epithelium in protecting the body from pathogens and environmental stressors. Among the factors affecting epithelial function, temperature variation along the respiratory tract has emerged as a key determinant in modulating cell differentiation and epithelial function.

This thesis aims to investigate the relationship between the temperature gradient and the cellular composition of the airway epithelium under homeostatic conditions. The underlying hypothesis of this study is that temperature variations along the respiratory tract induce specific changes at both the cellular and functional levels in the epithelium. Understanding this relationship could provide new therapeutic possibilities for the treatment of airway remodeling, a critical aspect of many chronic respiratory diseases, including asthma and chronic obstructive pulmonary disease (COPD). This research was conducted as part of my Master of Science in Biomedical Engineering at Delft University of Technology. The study was conducted under the supervision of Dr. Anne van der Does and Lars Wessels from the Department of Pulmonology at Leiden University Medical Center, and Dr. Massimo Mastrangeli from the Department of Microelectronics at Delft University of Technology.

Elena Ferraris
Delft, February 2025

ACKNOWLEDGEMENTS

First, I would like to thank Dr. van der Does, Prof. Dr. Hiemstra, and Dr. Mastrangeli for giving me the opportunity to work on this thesis, for their support, patience, and for trusting me with this project.

A special thanks to Lars and Amber: between a sudoku, a coffee, and our lunch walks, you taught me so much in the lab and supported me through every stage of this journey. I couldn't have done it without you!

To the whole PulmoScience Lab at LUMC in Leiden, thank you for always being so helpful and for all the great advice you have shared over these months.

To my study friends, Suvi and Eline, thank you for being by my side through the ups and downs, for sharing the struggles and the laughs, and for making these years unforgettable. I'll miss our days together so much!

To Hayo, Jasmijn, Yael, and Leon, my perfect partners in crime for all the crazy adventures, whether in the Netherlands or around the world. Thank you for witnessing all my emotional rollercoasters. Honestly, we could turn our endless voice messages into an audiobook!

To Marta, my high school friend, my travel buddy, and the friend I can always count on. Thank you for always going along with my crazy ideas without ever doubting me. No matter how far apart we were, you were always there for me, and I'll never stop being grateful for you!

To Cecilia, my childhood friend, thank you for always pushing me to go for what I wanted without fear. I wouldn't be the same person if we hadn't met back in kindergarten.

To all my housemates and friends who made the Netherlands feel like home, and to my Italian friends for always welcoming me back with open arms whenever I go back to Genova.

And last but not least, the biggest thank you to my parents, Cecia and Paolo. Without you, I wouldn't have made it. Thank you for always supporting me in every decision and for allowing me to discover and follow my passions, despite the distance.

Vi voglio bene!

Table of Contents

***Abstract*—The airway epithelium plays a crucial role in respiratory defense by regulating cellular composition and related functions to maintain pulmonary homeostasis. This study investigates the impact of temperature variations (33°C and 37°C) on cellular differentiation, mucociliary function, and host defense responses of human primary bronchial and nasal epithelial cells (hPBECs and hPNECs) cultured at the air-liquid interface. A reduction of well-differentiated cell cultures from 37°C to 33°C significantly increased goblet cell-related gene expression (*MUC5AC*) while decreasing ciliated cell-related gene expression (*FOXJ1*). Mucociliary clearance was generally impaired at 33°C, with ciliated cells displaying less organized ciliary alignment; however, donor variability made it challenging to establish consistent trends. Furthermore, cultures maintained at 33°C exhibited an enhanced antiviral response to Poly(I:C), as indicated by increased *IFNL* gene expression in both bronchial and nasal epithelial cells. In contrast, temperature had no effect on the inflammatory response to whole cigarette smoke in bronchial cultures but attenuated the oxidative stress response in nasal cultures, as evidenced by reduced *HMOX1* expression at 33°C. Analysis of Notch signaling revealed no significant alterations in the expression of key target genes (*HES1*, *HEY1*), with the exception of a transient decrease in *HEY2* at 33°C, suggesting complex regulatory interactions. These findings support the hypothesis that the temperature gradient along the airway tree influences the spatial distribution of airway epithelial cells, with potential implications for intervention with airway remodeling in chronic lung diseases, such as asthma and chronic obstructive pulmonary disease. However, further studies are needed to confirm these results and explore their clinical relevance.**

1 Introduction

The airway epithelium consists of a diverse group of cells that play crucial roles in protecting the respiratory system. The epithelial barrier acts as the primary defense mechanism against inhaled and airborne pathogens while simultaneously contributing to the regulation and preservation of pulmonary homeostasis. Its fundamental functions include the regulation of mucociliary clearance, maintenance of fluid homeostasis, and modulation of inflammatory responses triggered by tissue injury [1]. The cellular composition of the airway epithelium is characterized by several cell types and cellular subsets. The most prominent cell types include goblet cells, ciliated cells, basal cells, and club cells [2]. Their spatial distribution along the respiratory tract follows a gradient that reflects the varying properties and prevalence of each cell type along the proximal-distal axis, adapting to functional demands. In the proximal airways, the number of goblet and basal cells is greater than in the distal airways, where other epithelial cell types are predominant [3]. Goblet cells are essential for mucus secretion, which traps pathogens and particles, contributing to airway defense [1]. As part of their progenitor function, basal cells play key roles in the maintenance, regeneration, and repair of the airway epithelium [4]. In the distal airways, goblet and basal cell densities decrease, while club cell density progressively increases [3]. Like basal cells, club cells can further differentiate into specialized cell types and contribute to airway protection and maintenance. Recent research has shown that they can contribute to alveolar homeostasis, highlighting their multifunctional importance in respiratory health [5]. Finally, ciliated cells are distributed throughout the respiratory epithelium and facilitate mucociliary clearance through coordinated movement of their apical cilia [6].

The spatial distribution of different cell populations along the proximal-distal axis of the respiratory tree is regulated by various cues and mechanisms as reviewed in the attachment (Supplementary Literature Review). Several studies have identified temperature as a key factor in controlling the distribution patterns and cellular composition of the airway epithelium [7, 8]. In addition, studies have shown the presence of a well-defined thermal gradient along the airway, with higher temperatures in the distal region than in the proximal region [7, 9]. Notably, a temperature gradient ranging from 32°C to 35°C has been reported along the entire respiratory epithelium [9]. Similarly, an average temperature of 33°C in the nasal airways and 37°C in the smaller airways has been observed [7]. Unlike the luminal airway temperature measured in [9], which changed with the respiratory cycle, the mucosal temperature recorded in [7] remained relatively stable. Changes in the temperature properties along the proximal-distal tract can induce structural and functional adaptations in the airway epithelium. Notably, significant temperature changes can affect the morphology and function of airway epithelial cells, especially in ciliated cells and goblet cells [8, 10]. An increase in temperature is associated with a linear increase in ciliary beat frequency up to a maximum of 40°C, above which irreversible damage to ciliated cells occurs, including a reduction in ciliary beat frequency,

shortening of cilia, fusion of ciliary ends and, in some cases, cell degeneration [11]. Furthermore, high-temperature exposure also induces an increase in mucus production by goblet cells [12, 13]. Similarly, lower temperature exposure causes morphological and functional changes in epithelial cells, resulting in structural damage to ciliated cells and increased mucus secretion [14]. A previous study has also reported increased mucus secretion in response to low-temperature exposure; however, it remains unclear whether this increase is attributed to enhanced activity of individual goblet cells, an increase in their numbers, or to a greater release of accumulated mucus [15]. These findings suggest a possible correlation between the temperature gradient along the respiratory tree and the spatial distribution of airway epithelial cells, although this hypothesis needs further experimental confirmation. Although several studies have examined the effects of temperature on ciliated cell differentiation and mucus production by goblet cells, the impact of temperature in the distal airways and the relationship with the temperature gradient and airway epithelial cell composition remain largely unexplored. Understanding this relationship could provide new therapeutic possibilities for the treatment of airway remodeling, a critical aspect of many chronic respiratory diseases, including asthma and chronic obstructive pulmonary disease (COPD).

The objective of this study is to investigate the influence of temperature on the regulation of the cellular composition of the airway epithelium under homeostatic conditions. We hypothesized that temperature variations along the respiratory tree induce distinct cellular and functional changes in the airway epithelium. To test our hypothesis, we have used well-differentiated human primary bronchial epithelial cells (hPBECs), and human primary nasal epithelial cells (hPNECs) exposed to two different thermal conditions, 33°C and 37°C. Initially, the effects of the different temperatures on the cellular composition of the airway epithelium will be examined. The impact of temperature on the mucociliary function of ciliated cells will subsequently be studied, focusing on ciliary beat frequency and mucociliary clearance. In addition, the mechanism behind the temperature-driven effect will be observed. Finally, the influence of temperature on hPBECs and hPNECs subjected to pathogenic stimuli, such as cigarette smoke, will be assessed to understand changes in the immune response and barrier function of the epithelium.

2 Methods

2.1 Primary bronchial epithelial cell culture

To test our hypothesis, biobank-derived human primary bronchial epithelial cells (hPBECs) were isolated from bronchial rings obtained from patients undergoing lung cancer resection surgery at the Leiden University Medical Center (LUMC, Leiden, the Netherlands). The lung tissue was obtained from patients enrolled in the biobank through a no-objection system for coded, anonymous use in further research (www.coreon.org). The use of these biobank samples for research purposes was approved by the institutional medical ethical committee (BB22.006/AB/ab). Since 01-09-2022, patients have been enrolled in the biobank using written informed consent, in accordance with local regulations from the LUMC biobank, with approval from the institutional medical ethical committee (B20.042/KB/kb). The cells were cultured following a published protocol [16]. In summary, hPBECs were thawed and seeded in T75 flasks coated with a mixture of 30 µg/mL PureCol (Advanced BioMatrix, San Diego, CA, USA), 10 µg/mL bovine serum albumin (BSA, Fraction V; Thermo Fisher Scientific, Bleiswijk, Netherlands) and 5 µg/mL human fibronectin solution (CellSystems, Troisdorf, Germany). Cells were expanded in complete Keratinocyte SFM (serum free medium) (Thermo Fisher Scientific, Bleiswijk, Netherlands) until reaching 80-90% confluence within a week. Subsequently, 40.000 cells per insert, derived from a single donor, were seeded onto semi-permeable coated inserts. The cells were maintained in the insert in complete BD medium (cBD medium) on both the apical and basal sides of the inserts until complete confluency (100%) was achieved [16]. Upon reaching confluency, after approximately one week, the cells were exposed to an air-liquid interface (ALI) by removing the apical medium and replacing the basal medium with Air-Liquid Interface Medium (Promocell, Heidelberg, Germany) enriched with supplements according to the manufacturer's instructions. The culture medium was replaced three times a week, excess mucus and debris were removed by washing the

apical surface with warm PBS, and transepithelial electrical resistance (TEER) was measured weekly. After 10 days, ciliated cells were observed under the microscope, with complete differentiation occurring after three weeks of exposure to the air-liquid interface.

2.2 *Primary nasal epithelial cell culture*

Primary nasal epithelial cells (PNECs) were isolated by scraping epithelial cells from the nasal mucosa. The isolated cells were first expanded as organoids, then cultured on inserts under submerged conditions, and subsequently transferred to the air-liquid interface (ALI) using established protocols to generate fully differentiated mucociliary epithelial cultures. Briefly, hPNECs were seeded into organoids using airway organoid medium in basement membrane extract (BME). Upon reaching the organoid density, cells were dissociated and transferred to pre-coated permeable inserts at a seeding density of 120.000 cells per insert and maintained under submerged conditions until achieving full confluence (PneumaCult™-Ex Medium, StemCell Technologies, Canada). Once 100% confluence was reached, the apical medium was removed to establish an air-liquid interface (ALI), and the cells were maintained in ALI-maintenance medium (PneumaCult™, StemCell Technologies, Canada). The culture medium was replaced three times a week. To remove cellular debris and accumulated mucus, the apical surface was washed with warm PBS prior to each medium change, following a protocol analogous to that used for hPBECs. Transepithelial electrical resistance (TEER) was measured weekly to monitor barrier integrity. Unlike hPBECs, which require five weeks of ALI exposure to achieve complete maturation, hPNECs reach full maturation within three weeks. At this stage, both ciliated and goblet cells were fully differentiated and mature, characterized by the presence of functional motile cilia and mucus production.

2.3 *Study design*

Primary human bronchial epithelial cells and primary human nasal epithelial cells, differentiated at the air-liquid interface (ALI) for three weeks, were next incubated at 33°C or 37°C for three or seven days to evaluate the impact of temperature on cellular composition and functionality. At day 3 and 7 after the temperature change, cells were lysed, and RNA was isolated and stored for subsequent analysis of changes in gene expression of *FOXJ1*, *MUC5AC*, and *SCGB1A1* in cells exposed to the two different temperatures using RT-qPCR. Furthermore, to detect markers of ciliated and goblet cells, protein expression was assessed via immunofluorescence (IF) using specific antibodies against acetylated α-tubulin and Muc5AC. The percentage of cytotoxicity was evaluated within 48 hours post-treatment at both time points by measuring lactate dehydrogenase (LDH) release into the culture medium and comparing it to the maximum LDH release obtained through complete cell membrane disruption. This approach enables the distinction between LDH release specifically induced by temperature changes and the maximal possible release, ensuring an accurate interpretation of the data. Additionally, ciliary beat frequency (CBF) and mucociliary clearance (MCC) were measured to assess the impact of temperature on ciliary function and, consequently, on the ability of ciliated cells to perform mucociliary clearance.

To assess the effect of temperature on primary bronchial epithelial cells and primary nasal epithelial cells previously exposed to pathogenic stimuli, differentiated cells were treated with Poly(I:C), a synthetic analog of double-stranded RNA (dsRNA) that mimics an virus infection, or exposed to whole cigarette smoke (WCS) using 3R4F reference cigarettes (University of Kentucky, Lexington, KY, USA) [17]. In the first experimental condition, differentiated bronchial epithelial cells and nasal epithelial cells cultured at the air-liquid interface (ALI) were incubated at 33°C or 37°C for seven days. Subsequently, they were apically treated with 10 µg/mL of Poly(I:C) to evaluate the effect of temperature at 6- and 24-hours post-treatment. To control the specific effects of Poly(I:C), two control conditions were included: a well with only cells to account for baseline measurements (NC) and a well containing the cells and PBS to differentiate nonspecific responses potentially arising from changes in well conditions. Additionally, the transition from 33°C to 37°C was examined by maintaining the well-differentiated cells at 33°C for one week, followed by exposure to a viral trigger and subsequent incubation at 37°C for 6

or 24 hours. This approach was used as a control to exclude that the observed changes in gene expression were attributable to culturing at a lower temperature during the treatment exposure. In the second experimental condition, differentiated cells were incubated under the same conditions (33°C or 37°C for seven days) and subsequently exposed to whole cigarette smoke (WCS) or air (AIR) in specialized exposure chambers. In these chambers, ALI-PBECs and ALI-PNECs were exposed to WCS generated from a single cigarette, delivering approximately 2 mg of cigarette smoke particles. Smoke infusion was achieved using a mechanical pump with a continuous airflow rate of 1 L/min over a 5-minute period. Residual smoke was ventilated with room air for 10 minutes after exposure. The effect of smoke exposure was subsequently evaluated at 4- and 24-hours post-exposure under the conditions of 37°C, 33°C, and the transition from 33°C to 37°C. To investigate the mechanism underlying the effect of temperature, the Notch signaling pathway was analyzed, as it plays a crucial role in regulating cell differentiation and proliferation in the airway epithelium [18]. Specifically, the gene expression of its key target genes (*HES1*, *HEY1*, and *HEY2*) was evaluated using RT-qPCR in bronchial epithelial cells exposed to two different temperatures for three and seven days, to determine whether temperature can modulate the balance between the proliferation and differentiation of ciliated and goblet cells.

2.4 RNA isolation and quantitative PCR (qPCR)

RNA from bronchial and nasal epithelial cells, lysed in RNA lysis buffer (Promega Benelux B.V., Leiden, The Netherlands), was extracted using the Maxwell RSC SimplyRNA Tissue Kit (Promega Benelux B.V., Leiden, The Netherlands) and quantified with the Nanodrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA synthesis was carried out using oligo(dT)15 primers (Qiagen Benelux, Venlo, The Netherlands) and M-MLV Reverse Transcriptase (Promega Benelux B.V., Leiden, The Netherlands), in the presence of an RNase inhibitor (RNAsin, Promega Benelux B.V.) and a dNTP mix (Promega Benelux B.V., Leiden, The Netherlands). Quantitative real-time PCR (qPCR) was conducted in triplicate on the CFX384 Real-Time PCR system (Bio-Rad) following a standard protocol, including melting curve analysis (65–95°C), using iQ SYBR Green Supermix (Bio-Rad). The primers listed in Supplementary Table 1 were analyzed using *RPL22* and *OAZ1* as reference genes, previously selected according to the Genorm method (Primer Design, Southampton, UK). Data analysis was performed using the Bio-Rad CFX Manager 3.0 software (Bio-Rad).

2.5 LDH assay

The cytotoxicity assay was performed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Benelux B.V., Leiden, The Netherlands). Cells were stimulated with appropriate treatments, and a positive control, consisting of 0.1% Triton X-100 diluted in PBS, was included to assess the maximum LDH release.

2.6 Immunofluorescence staining

Immunofluorescence staining was performed on human primary bronchial epithelial cells (hPBECs) and human primary nasal epithelial cells (hPNECs) cultured at the air-liquid interface (ALI) to visualize specific cellular targets using confocal microscopy. Prior to staining, cultures were fixed with 4% paraformaldehyde (PFA) for 10 minutes at 4°C and washed with PBS at room temperature. Blocking was performed using PBS containing 1% BSA and 0.3% Triton X-100 for 10 minutes at room temperature (RT). Primary antibodies, diluted in the blocking solution, were applied to the membrane containing the cells, which were excised from the inserts using a scalpel blade (Supplementary Table 2). The filter sections were placed cell-side down on 30 µL drops of antibody solution for 1 hour at RT. After washing with PBS, Alexa Fluor-conjugated secondary antibodies (1:200 dilution) and DAPI (1:500 dilution) were added in blocking solution and incubated for 30 minutes at RT in the dark. Filters were washed with PBS and dH2O, air-dried, and mounted onto slides using ProLong Gold Antifade reagent. Coverslips were applied, allowed to stabilize overnight, and stored in the dark at RT. Image acquisition was carried out using a Leica DMi8 inverted microscope (Leica Microsystems, Wetzlar, Germany) equipped with an Andor Dragonfly 200 spinning disk confocal (Andor Technology, Belfast, UK) and Imaris Viewer (Oxford Instruments, Abingdon, UK) software.

2.7 *Ciliary beat frequency*

Ciliary beating frequency (CBF) was assessed using an Andor Dragonfly 200 microscope (Andor Technology, Belfast, UK) equipped with a 40x water immersion objective. Primary epithelial cells were washed with warm PBS for at least 10 minutes to remove mucus. The membrane from the insert containing the cells was excised, inverted, and placed in a µ-image dish. Four drops of culture medium were applied to the membrane, which was then covered with a coverslip. Image acquisition involved recording 10-second videos at 40x magnification, with the light polarizer adjusted to enhance cilia visualization. Quantification of CBF can be performed using MATLAB script.

2.8 *Mucociliary clearance measurement*

Mucociliary clearance (MCC) was evaluated by washing the samples with warm PBS, followed by the addition of 100 µL of 1 µm fluorescent polystyrene microspheres (ThermoFisher Scientific) diluted 1:100 in ALI medium. The samples were maintained at 37°C in a heat chamber throughout the experiment. The fluorescent particles facilitated the visualization of tracer movement and, consequently, ciliary activity over time. Imaging was performed using a confocal microscope equipped with an Andor Dragonfly 200 spinning disk confocal system (Andor Technology, Belfast, UK), a 10x objective lens, and appropriate filters. Videos of five fields of view per sample were recorded over a 10-second interval with a temporal resolution of 20–30 frames per second. Video data were analyzed in ImageJ-Fiji using the Z-projection function with the maximum intensity setting, which consolidated stack data into a single two-dimensional image highlighting the brightest structures. Potential artifacts (e.g., lines) were examined, and processed images were exported for subsequent analysis. While precise quantification required a MATLAB script, ImageJ-generated images provided a reliable preliminary assessment in cases with visually apparent differences.

2.9 *Statistical analysis*

Statistical analyses were conducted using GraphPad Prism 10.2.3 (GraphPad Software Inc., La Jolla, CA, USA). Data were evaluated with T-tests or one-/two-way ANOVA, and differences were considered statistically significant at p-values < 0.05.

3 Results

3.1 *Temperature-related changes in the composition of airway epithelial cells*

To investigate whether spatial differences in cellular composition within the respiratory tract are driven by the existing temperature gradient, we evaluated the impact of a temperature reduction, resembling nasal region-relevant conditions on the cell-type composition of airway epithelial cultures. Cytotoxicity analysis revealed that cells effectively maintained membrane integrity at both 37°C and 33°C (Supplementary Fig. 1) . Subsequently, we assessed the cellular composition of the cultures by analyzing the gene expression of airway epithelial cell-type specific genes and performing immunofluorescence staining. After three weeks of differentiation, a subset of cultures was switched from 37°C to 33°C for up to three and seven days. Three days after this temperature switch, expression of the goblet cell-related gene (*MUC5AC*) was higher in cultures maintained at 33°C than at 37°C, except for one donor that showed reduced expression at 33°C (Fig.1A). However, the difference observed between the two temperature conditions did not show statistical significance. Instead, an opposite trend was observed for the ciliated cell-related gene (*FOXJ1*), with decreased gene expression in cultures maintained at 33°C compared to 37°C, except for one donor, whose culture exhibited the opposite trend (Fig.1A). Notably, the donors identified as outliers for *MUC5AC* expression were not the same individuals as those exhibiting outlier behavior for *FOXJ1* expression. Similarly, these differences between temperature conditions were not statistically significant. After one week of exposure to two different temperatures, bronchial epithelial cell cultures from six donors maintained at 33°C exhibited significantly higher expression of the goblet cell-related gene (*MUC5AC*) compared to those maintained at 37°C (Fig.1B). The expression of the ciliated cell-related gene (*FOXJ1*) revealed significant differences between the two experimental conditions (Fig.1B). In contrast to what was observed for goblet cells, the expression *FOXJ1* showed a uniform reduction across all six donors as the temperature decreased.

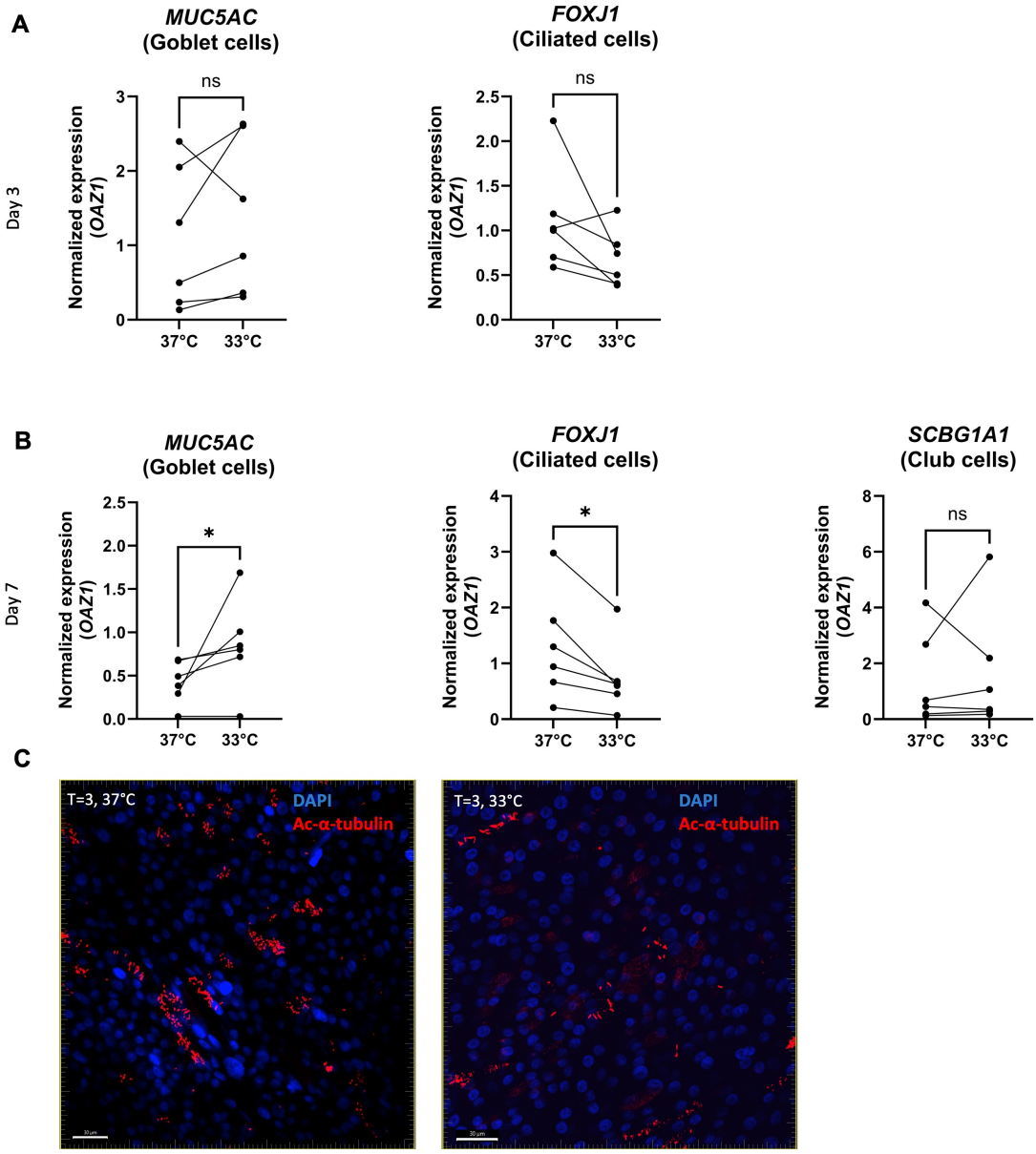


Fig. 1. Effect of temperature changes on bronchial epithelial cell composition at day 3 and 7 after the temperature shift. **A**. Gene expression in ALI-PBECs after three days following the temperature shift. Expression of the goblet cell-associated gene (*MUC5AC*), normalized to *OAZ1*, at 37°C and 33°C (left). Expression of the ciliated cell-associated gene (*FOXJ1*), normalized to *OAZ1*, at 37°C and 33°C (right). No significant differences were detected (ns) using the Wilcoxon matched-pairs signed rank test. N = 6 donors. **B**. Gene expression in ALI-PBECs after seven days following the temperature shift. Expression of the goblet cell-associated gene (*MUC5AC*), normalized to *OAZ1*, was significantly increased at 33°C compared to 37°C ($p < 0.05$, Wilcoxon matched-pairs signed rank test) (left). Expression of the ciliated cell-associated gene (*FOXJ1*), normalized to *OAZ1*, was significantly decreased at 33°C compared to 37°C ($p < 0.05$, Wilcoxon matched-pairs signed rank test) (middle). Expression of the club cell-associated gene (*SCGB1A1*), normalized to *OAZ1*, showed no significant difference between 37°C and 33°C (Wilcoxon matched-pairs signed rank test) (right). N = 6 donors. **C**. Representative immunofluorescence staining of ciliated cells using acetylated α-tubulin as a marker, three days after the temperature shift. Cultures were maintained at 37°C (left) or shifted to 33°C (right). Images illustrate the distribution of acetylated α-tubulin (red) highlighting the cilia of ciliated cells, with nuclei counterstained using DAPI (blue). Scale bar: 30 µm

Conversely, the expression of club cell-related gene (*SCGB1A1*) exhibited no consistent pattern across donors, and no significant differences were observed between the two experimental conditions (Fig.1B). This variability was evident as the cultures of two donors showed a decrease in gene expression at 33°C, while those of the other four exhibited an increase.

Immunofluorescence staining analysis confirmed the findings obtained from the gene expression analysis of airway epithelial cell-specific genes. Specifically, three days after the temperature shift, a reduction in the presence of cilia was observed in most cultures maintained at 33°C compared to those maintained at 37°C (Fig.1C). Additionally, cilia in the 33°C condition appeared less organized and aligned. An opposite trend was noted in one donor culture, while another donor culture showed no differences between the two temperature conditions, which could be attributed to delayed cell differentiation or potential technical artifacts during the staining process (Supplementary Fig. 2). Moreover, goblet cells were not detected in any of the cultures, which could be attributed to staining artifacts or technical limitations.

When we repeated the experiment with well-differentiated nasal epithelial cells from a single donor, a very mild increase in the expression of goblet-cell could be observed upon switch to 33°C, while a similarly mild reduction of ciliated cell-related gene expression could be observed after one week of exposure (Fig.2A). These results confirm the observed effects in PBECs, however must be interpreted with care because of the single donor used. Notably, these changes in gene expression were less pronounced compared to those observed in bronchial epithelial cells. Immunofluorescence staining showed reduced ciliary density in nasal epithelial cell cultures maintained at 33°C compared with those maintained at 37°C, thereby supporting

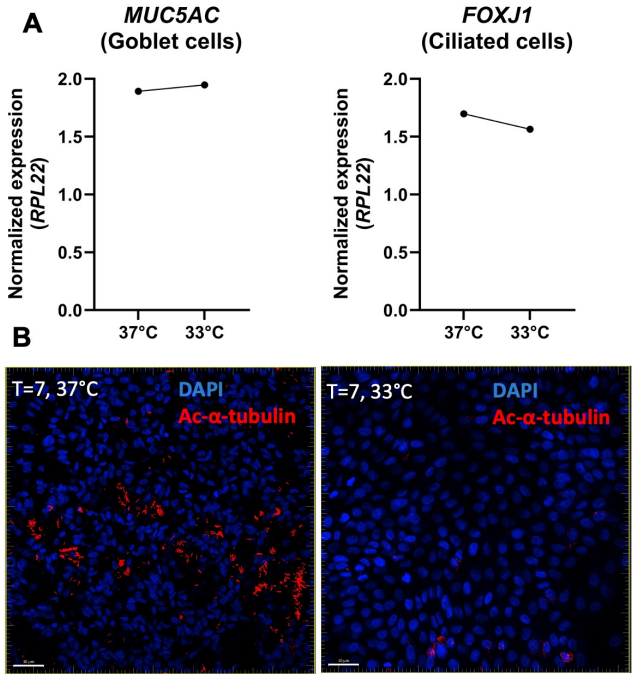


Fig. 2. Effect of temperature changes on nasal epithelial cell composition. **A**. Gene expression in ALI-PNECs after seven days following the temperature shift. ALI-PNECs were differentiated for three weeks before some cultures were maintained at 37°C, while others were shifted to 33°C for seven days. Expression of the goblet cell-associated gene (*MUC5AC*), normalized to *RPL22*, at 37°C and 33°C (left). Expression of the ciliated cell-associated gene (*FOXJ1*), normalized to *RPL22*, at 37°C and 33°C (right). N = 1 donor. **B**. Representative immunofluorescence staining of ciliated cells using acetylated α-tubulin as a marker on day 7 after the temperature shift. Cultures were maintained at 37°C (left) or shifted to 33°C (right). Images illustrate the distribution of acetylated α-tubulin (red) highlighting the cilia of ciliated cells, with nuclei counterstained using DAPI (blue). Scale bar: 30 µm

the results obtained from gene expression analysis of ciliated cell-related gene (Fig.2B). However, interpretation of the data must be approached with care because the images analyzed were obtained from single-donor nasal epithelial cell cultures.

Overall, these results show how temperature gradients influence cellular composition and related gene expression of airway epithelial cells, with potential implications for mucociliary clearance and mucus production.

3.2 Influence of temperature on mucociliary functioning and ciliary beating alignment

To evaluate the influence of temperature on mucociliary functioning (MCC) of ciliated cells, we monitored the displacement of fluorescent microbeads over the epithelial surface at 37°C. Specifically, on day 3, ciliary movement in ciliated cells was not detectable in some donors under both temperature conditions (37°C and 33°C) (Supplementary Fig. 3). While certain donors exhibited improved ciliary alignment when previously cultured at 37°C compared to 33°C, others showed the opposite trend. The variability between donors makes it complicated to make conclusions about mucociliary function three days after the temperature change. Additionally, MCC was measured on day 7 after the temperature change to assess potential similarities with previously observed effects (Supplementary S3). On day 7, cell cultures maintained at 37°C exhibited an accelerated MCC function compared to those maintained at 33°C (Fig.3B). However, since no measurements were performed on the day of the switch, it cannot be excluded that the observed differences are partially due to reduced function at 33°C rather than actual acceleration at 37°C. In addition, the opposite trend was observed in other donors, making it challenging to draw definitive conclusions (Fig.3A). Finally, in some cultures at

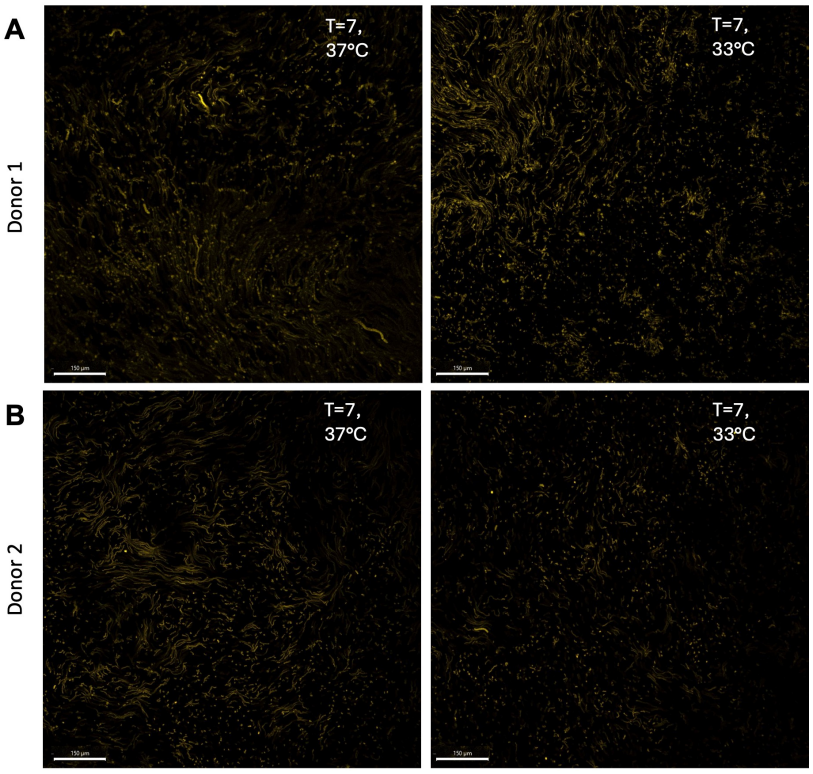


Fig. 3. Representative images of fluorescent bead displacement in bronchial epithelial cell cultures from different donors. **A**. Fluorescent bead displacement in bronchial epithelial cell cultures maintained at 37°C, assessed on day 7 after the temperature change (left). Fluorescent bead displacement in cultures from the same donor maintained at 33°C, assessed on day 7 after the temperature change (right). **B**. Fluorescent bead displacement in bronchial epithelial cell cultures from a second donor maintained at 37°C, assessed on day 7 after the temperature change (left). Fluorescent bead displacement in cultures from the same donor maintained at 33°C, assessed on day 7 after the temperature change (right). Scale bar: 150 µm

37°C, ciliary beating displayed greater alignment, while in cultures at 33°C, organized alignment was less evident, and ciliary movement appeared random. Once again, inter-donor variability was observed, further complicating the identification of a clear trend (Supplementary Fig. 3). Overall, these observations suggest that, in some donors, a reduction in temperature is associated with a slowing of ciliary cell movement, potentially indicating a delay in the processes that promote the functional maturation of airway epithelium.

3.3 Impact of temperature change on the response to Poly(I:C) by epithelial cells

We subsequently investigated how changes in temperature affect the response of bronchial and nasal epithelial cells to a pathogenic stimulus, specifically Poly(I:C), a synthetic analog of double-stranded RNA (dsRNA), used to mimic a virus infection. Cell cultures were treated with this viral trigger seven days after the temperature change, with exposure durations of 6 and 24 hours.

In bronchial epithelial cell cultures, *IFNL* gene expression was assessed in response to Poly(I:C), after maintaining the cell cultures at 33°C for seven days. To ensure that changes in gene expression were not solely due to the lower temperature during Poly(I:C) exposure, potentially impairing cellular responses, we included a control in which cells were incubated at 33°C for seven days but shifted to 37°C during exposure to Poly(I:C). In the negative control groups treated with PBS alone (solvent control), *IFNL* expression was nearly absent or negligible across all temperature conditions. The Poly(I:C)-treated groups demonstrated a marked induction of *IFNL* gene expression. Notably, the enhanced antiviral response observed at 33°C is specifically attributable to the low temperature, as gene expression levels in the transition condition (33°C $\rightarrow$ 37°C) reverted to those observed at 37°C after 6 hours of exposure (Fig.4A). After 24 hours of exposure, gene expression levels appeared higher in cultures maintained at 37°C compared to those maintained at 33°C (Fig.4A). However, this difference was primarily driven by the presence of an outlier. When this outlier was excluded, the antiviral response at 24 hours remained more pronounced in cultures maintained at 33°C, aligning with the trend observed at 6 hours Fig. 4. However, statistical analysis revealed no significant differences in expression levels between the temperature conditions (37°C and 33°C) at either exposure duration (6 or 24 hours), as indicated in Fig.4A and Fig.4B. After 6 hours, *IFNL* gene expression increased in response to Poly(I:C) exposure, with higher gene expression levels at a temperature of 33°C, although variability was evident between donors (Fig.4C). Specifically, for almost all donors, gene expression at 33°C was higher compared to 37°C. After 24 hours of treatment exposure, the gene expression pattern remained consistent with previous observations, with the highest expression levels detected in cultures maintained at 33°C in almost all donors (Fig.4D). In both exposure time points, a single donor exhibited an opposite trend compared to the others, identifiable as an outlier. However, even after excluding the outlier, no significant differences were observed, as shown in the Supplementary Fig. 4. These findings, consistent at both 6 and 24 hours of exposure, indicate that cultures maintained at 33°C exhibit an enhanced antiviral response to Poly(I:C) compared to those at 37°C, although this difference does not reach statistical significance.

Unlike the gene expression of *IFNL*, which is absent under control conditions due to the lack of viral stimuli, *CXCL8* expression was detectable in both baseline and exposed cultures at all time points (6 and 24 hours) (Fig.4E & Fig.4F). After 6 hours, *CXCL8* expression was elevated in the Poly(I:C)-treated cultures compared to the NC and PBS groups, indicating an acute inflammatory response to the stimulus (Fig.4E). However, no significant differences were observed between the 37°C, 33°C, and 33°C $\rightarrow$ 37°C conditions. After 24 hours, *CXCL8* expression exhibited a similar pattern to that observed at 6 hours (Fig.4F). However, a reduction in gene expression was noted in the 33°C and transition groups treated with Poly(I:C), indicating a reduction of the inflammatory response over time. Nonetheless, variability persisted among the Poly(I:C)-treated cultures, with some cultures still exhibiting elevated expression levels. Overall, these findings suggest that treatment with Poly(I:C) induces a transient increase in *CXCL8* expression, which is particularly evident at 6 hours, but is not affected by changes in temperature exposure.

A similar pattern was observed in nasal epithelial cells for both genes. The normalized expression of *IFNL* was nearly undetectable in both untreated

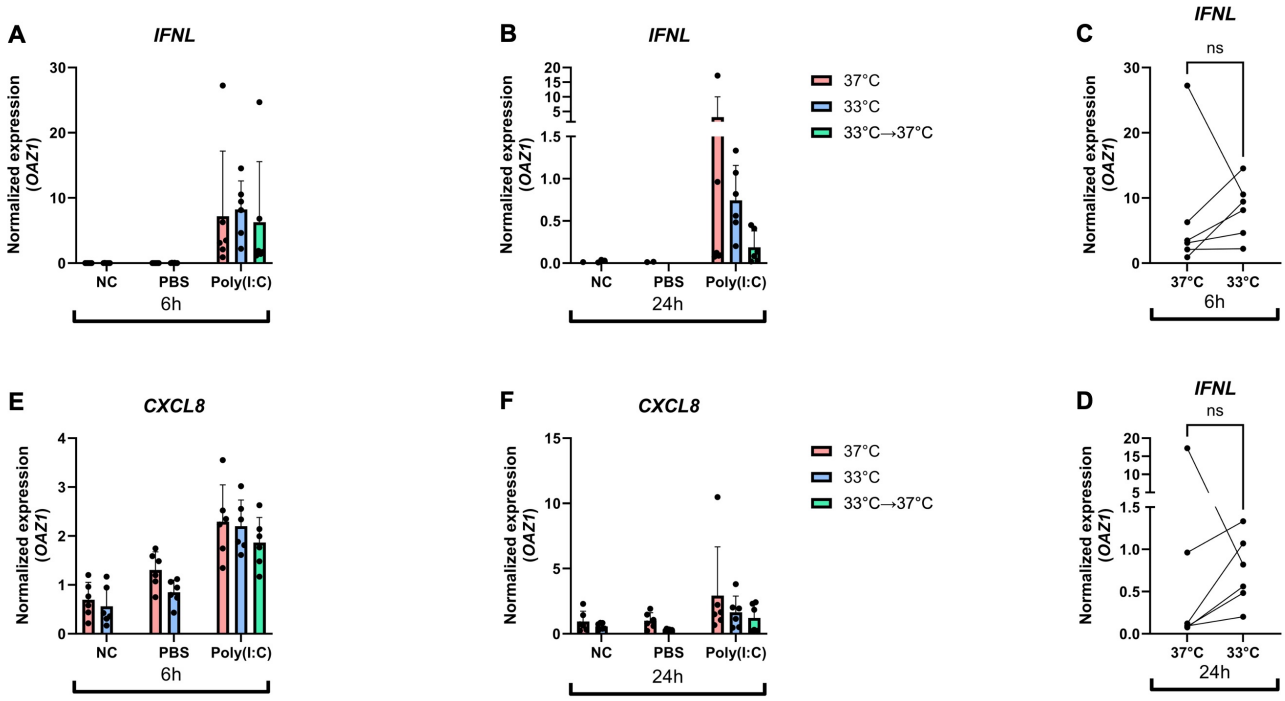


Fig. 4. Effect of temperature changes on the inflammatory gene expression in ALI-PBECs. **A**. Gene expression of *IFNL* normalized to *OAZ1* after 6 hours of exposure in the NC, PBS, and Poly(I:C) treatment groups at 37°C, 33°C, and 33°C → 37°C. No significant differences (ns) were observed between conditions. One outlier was present in this dataset. N = 6 donors. **B**. Gene expression of *IFNL* normalized to *OAZ1* after 24 hours of exposure in the NC, PBS, and Poly(I:C) treatment groups at 37°C, 33°C, and 33°C → 37°C. No significant difference was observed. One outlier was present in this dataset. N = 6 donors. **C**. Paired *IFNL* expression at 37°C and 33°C after 6 hours of exposure, normalized to *OAZ1*. No significant difference was observed using the Wilcoxon matched-pairs signed rank test. One outlier was present in this dataset. N = 6 donors. **D**. Paired *IFNL* expression at 37°C and 33°C after 24 hours of exposure, normalized to *OAZ1*. No significant difference was observed using the Wilcoxon matched-pairs signed rank test. One outlier was present in this dataset. N = 6 donors. **E**. Gene expression of *CXCL8* normalized to *OAZ1* after 6 hours of exposure in the NC, PBS, and Poly(I:C) treatment groups at 37°C, 33°C and 33°C → 37°C. No significant differences were observed. N = 6 donors. **F**. Gene expression of *CXCL8* normalized to *OAZ1* after 24 hours of exposure in the NC, PBS, and Poly(I:C) treatment groups at 37°C, 33°C and 33°C → 37°C. No significant differences were observed. N = 6 donors.

(NC) and PBS-treated cells across all experimental conditions (Fig.5A). This confirms that the induction of *IFNL* is specific to Poly(I:C) treatment and is not influenced by negative controls or temperature conditions in the absence of stimulation. When cells were treated with Poly(I:C), a clear temperature-dependent effect on *IFNL* induction was observed. At 33°C, *IFNL* expression was markedly higher compared to other conditions. In contrast, at 37°C, *IFNL* induction was reduced, indicating that physiological temperature may limit the response to Poly(I:C). To assess whether the increased response at 33°C is temperature-specific or due to other factors (e.g., cellular metabolism), a transition condition (33°C → 37°C) was included. Cells cultured at 33°C for one week and shifted to 37°C during exposure showed *IFNL* expression levels similar to those directly treated at 37°C. This suggests that the enhanced expression at 33°C is specifically attributable to the low temperature, as the shift to 37°C effectively resets the cellular response to a level comparable to that at physiological temperature. These results demonstrate that culture temperature modulates the Poly(I:C)-induced antiviral

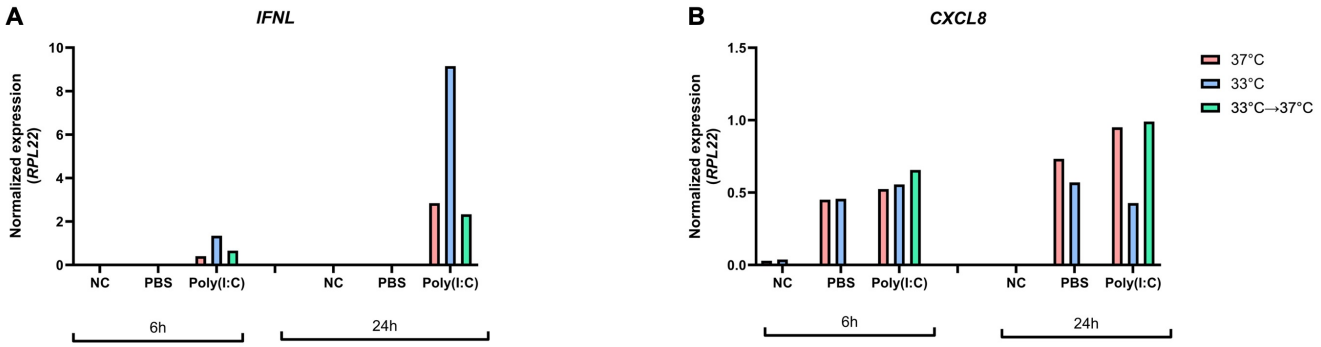


Fig. 5. Temperature effects on *IFNL* and *CXCL8* expression over time in ALI-PNECs exposed to a viral trigger. **A**. Normalized expression of *IFNL*, relative to *RPL22* in NC, PBS, and Poly(I:C) treatment groups at 37°C, 33°C, and after the temperature shift from 33°C to 37°C (33°C $\rightarrow$ 37°C). Data are shown for 6-hour and 24-hour time points. A marked increase in *IFNL* expression is observed in the Poly(I:C) group at 24 hours, particularly at 33°C. N = 1 donor. **B**. Normalized expression of *CXCL8*, relative to *RPL22*, in NC, PBS, and Poly(I:C) treatment groups under the same conditions (37°C, 33°C, 33°C $\rightarrow$ 37°C). *CXCL8* expression decreases after 6 and 24 hours of exposure in the Poly(I:C) group across 33°C. N = 1 donor.

response, with 33°C amplifying *IFNL* expression and increasing sensitivity to stimulation.

Regarding *CXCL8* expression, an increase was observed after 6 hours in both PBS- and Poly(I:C)-treated groups compared to the negative control (NC) across all temperature conditions (Fig.5B). Notably, the effect of Poly(I:C) was more pronounced compared to PBS, leading to a stronger induction of gene expression. After 6 hours, no significant differences were observed between the groups treated with Poly(I:C) at 33°C and 37°C, indicating the absence of a temperature-specific response during the initial phase of stimulation. However, within 24 hours, the Poly(I:C)-treated groups at 33°C showed lower *CXCL8* expression levels compared to those at 37°C (Fig.5B). This decrease in expression at 33°C suggests a potential accelerated downregulation of the inflammatory response at lower temperatures, as the transition group (33°C $\rightarrow$ 37°C) showed similar expression levels to those observed at 37°C. These results demonstrate that cultures maintained at 33°C may show a lower inflammatory response, compared with cultures maintained at 37°C, as evidenced by *CXCL8* gene expression. Together, these results indicate that temperature modulates antiviral but not inflammatory responses in bronchial epithelial cells, whereas it affects both in nasal epithelial cells.

3.4 Impact of temperature change on the inflammatory response to whole cigarette smoke by epithelial cells

To assess the impact of temperature on bronchial and nasal epithelial cell response to cigarette smoke, we analyzed the expression of genes related to oxidative stress (*HMOX1*) and inflammation (*CXCL8*).

In bronchial epithelial cells, the normalized expression of *HMOX1* was evaluated under different temperature conditions following exposure to whole cigarette smoke (WCS) (Fig.6A). Under control conditions (AIR), *HMOX1* expression was negligible across all tested temperatures, indicating the absence of baseline oxidative stress. Conversely, exposure to WCS resulted in a significant increase in *HMOX1* expression across all temperature conditions. These results indicate that WCS acts as an inducer of oxidative stress in bronchial epithelial cells, while temperature variations did not significantly modulate this response in bronchial epithelial cells.

The expression of *CXCL8* was also analyzed in bronchial epithelial cells exposed to whole cigarette smoke exposure (WCS) at both temperatures (33°C and 37°C) (Fig.6B). After 4 hours, *CXCL8* expression levels were higher compared to the AIR control under both temperature conditions. However, no significant differences were observed between the two temperatures, suggesting that the induction of

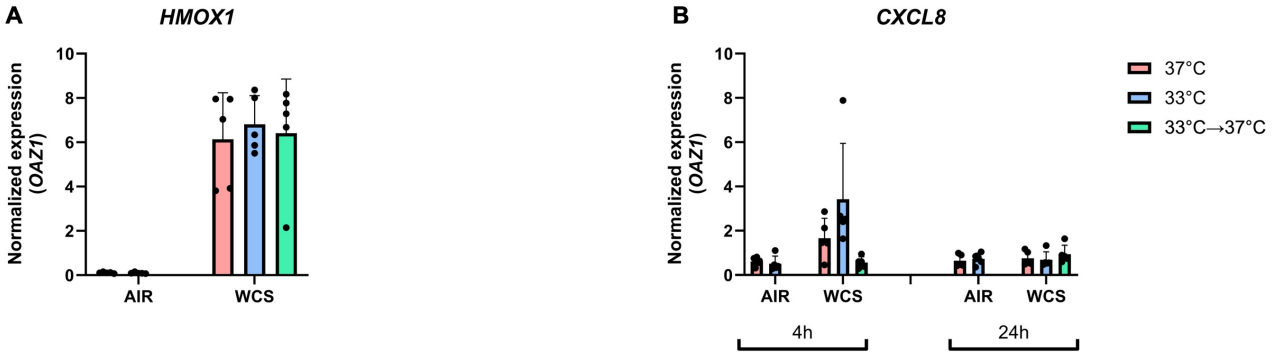


Fig. 6. Temperature effects on *HMOX1* and *CXCL8* expression over time in ALI-PBECs exposed to WCS. **A**. Normalized expression of *HMOX1* relative to *OAZ1* in AIR and WCS treatment groups under two temperature conditions (37°C, 33°C). Data are shown for 4-hour time point. No significant differences were observed between conditions. N = 6 donors **B**. Normalized expression of *CXCL8* relative to *OAZ1* in AIR and WCS treatment groups under two temperature conditions (37°C, 33°C). *CXCL8* expression increases after 4 and 24 hours of exposure in the WCS group, particularly at 33°C. N = 6 donors.

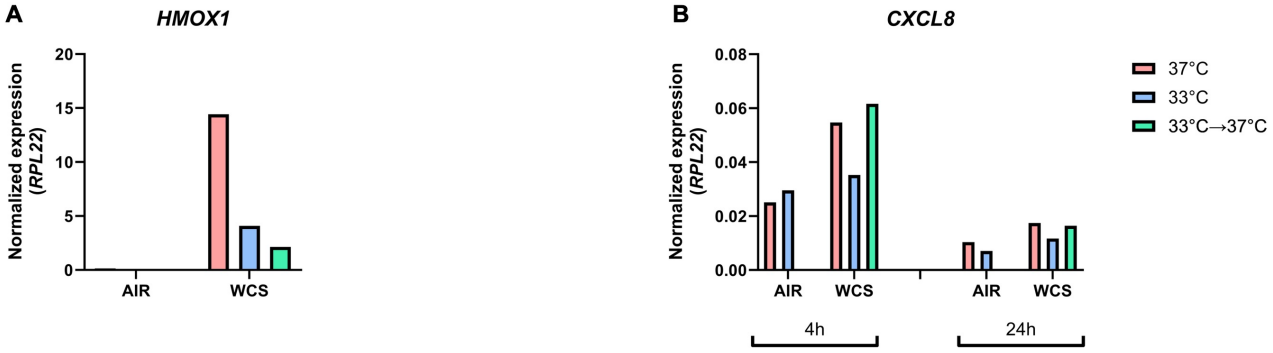


Fig. 7. Temperature effects on *HMOX1* and *CXCL8* expression over time in ALI-PNECs exposed to WCS. **A**. Normalized expression of *HMOX1* relative to *RPL22* in AIR and WCS treatment groups at 37°C, 33°C, and after switching from 33°C to 37°C (33°C → 37°C). Data are presented at 4 hours after exposure. A marked increase in *HMOX1* expression is observed in the WCS group at 37°C. N = 1 donor. **B**. Normalized expression of *CXCL8* relative to *RPL22* in AIR and WCS treatment groups under three conditions (37°C, 33°C, 33°C → 37°C). Data are presented at 4 hours and 24 hours. *CXCL8* expression shows a decrease after 4 hours of exposure in the WCS group across 33°C. N = 1 donor.

CXCL8 is not influenced by temperature variation. At 24 hours, *CXCL8* expression levels were reduced compared to those at 4 hours. Consistent with the results observed at 4 hours, no significant differences were noted between cultures maintained at 33°C and those observed at 37°C. The similarity in expression levels between the two temperatures at both exposure times suggests that temperature variation does not modulate the expression of genes associated with inflammatory responses to WCS.

We subsequently investigated the responses of nasal epithelial cells following WCS exposure to both temperatures (Fig.7A). As expected, in the AIR control cultures, *HMOX1* expression was undetectable, irrespective of the temperature applied. At 37°C, WCS exposure markedly induced *HMOX1* expression compared to the AIR control, highlighting its stimulatory effect at physiological temperature. Conversely, at 33°C, *HMOX1* expression in response to WCS was lower compared to 37°C, indicating that reduced temperature markedly attenuates the cellular response to WCS treatment. In the transition

condition (33°C → 37°C), *HMOX1* expression was lower than that observed at 33°C, suggesting that the shift to 37°C not only fails to restore gene expression but may further suppress it compared to the response at 33°C. These results indicate that the reduction in expression observed at 33°C cannot be attributed exclusively to long-term temperature effects alone.

CXCL8 expression after 4 hours was higher in WCS-exposed cultures compared to the AIR control group (Fig.7B). Specifically, culturing at 37°C induced higher expression levels than at 33°C. Notably, in the 33°C → 37°C transition condition, *CXCL8* expression levels were comparable to those observed at 37°C, suggesting that the response at 33°C is unique to that temperature and does not persist once the culture is shifted to 37°C. After 24 hours, *CXCL8* expression was drastically reduced across all conditions, although a slight response remained detectable in WCS-exposed cultures. These findings suggest that *CXCL8* induction is acute and highly sensitive to temperature variations, particularly during the first four hours of exposure.

Overall, these results highlight the impact of temperature on the response of bronchial and nasal epithelial cells to cigarette smoke exposure. While bronchial epithelial cells showed a consistent oxidative stress and inflammatory response to WCS regardless of temperature, nasal epithelial cells showed increased sensitivity to temperature changes, as evidenced by reduced expression of *HMOX1* and *CXCL8* at 33°C.

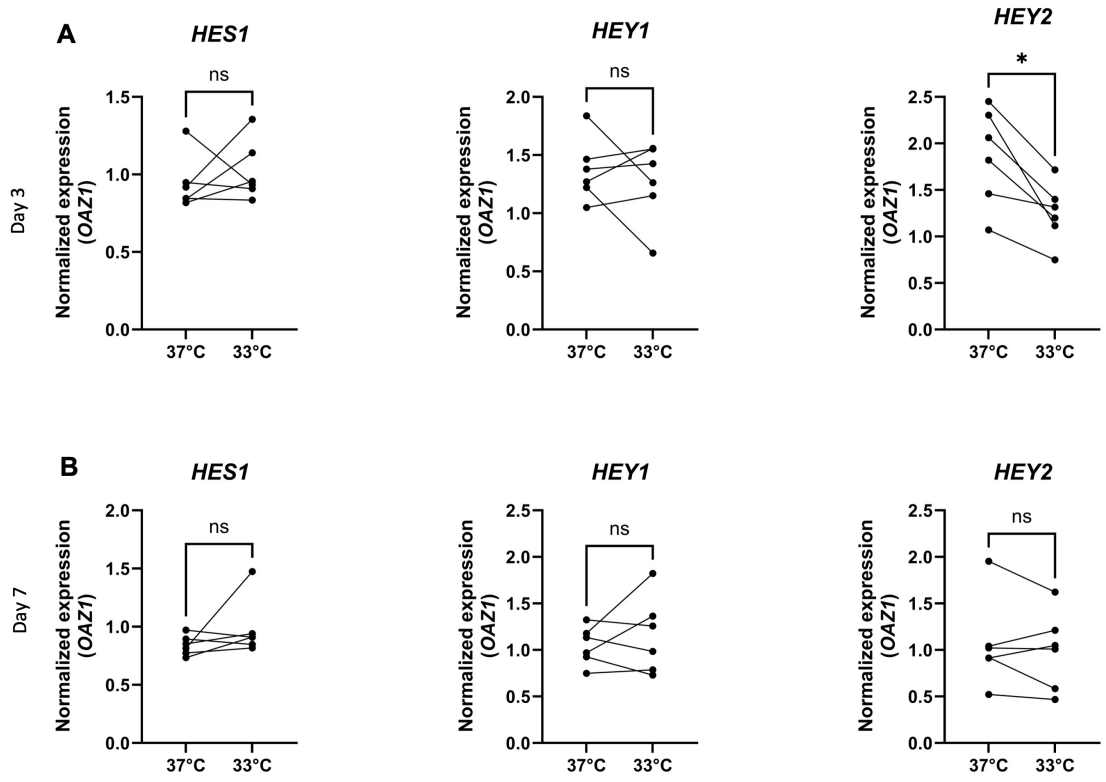


Fig. 8. Effect of temperature changes on the expression of Notch target genes (*HES1*, *HEY1*, *HEY2*) **A**. Gene expression three days after the temperature shift. Expression of *HES1* normalized to *OAZ1* at 37°C and 33°C (left). Expression of *HEY1* normalized to *OAZ1* at 37°C and 33°C (middle). Expression of *HEY2* normalized to *OAZ1* at 33°C was significantly lower compared to 37°C ($p < 0.05$, Wilcoxon matched-pairs signed rank test) (right). No significant differences (ns) were observed for *HES1* and *HEY1*. N = 6 donors. **B**. Gene expression seven days after the temperature shift. Expression of *HES1* normalized to *OAZ1* at 37°C and 33°C (left). Expression of *HEY1* normalized to *OAZ1* at 37°C and 33°C (middle). Expression of *HEY2* normalized to *OAZ1* at 37°C and 33°C (right). No significant differences were observed for any of the genes analyzed (Wilcoxon matched-pairs signed rank test). N = 6 donors.

3.5 The effect of temperature on Notch pathway target genes: analysis of HES1, HEY1, and HEY2

We investigated the effect of temperature variations on the activation of the Notch signaling pathway, which plays a key role in the regulation of differentiation and proliferation in airway epithelium. Specifically, it plays an important role in the differentiation toward goblet and ciliated cells. We therefore examined the expression of *HES1*, *HEY1*, and *HEY2*, key target genes of the Notch pathway, to evaluate the impact of temperature on these genes in bronchial epithelial cells. No statistically significant differences in *HES1* and *HEY1* gene expression were observed between the two temperature conditions at either time point (Fig.8A & Fig.8B). These findings suggest that this Notch pathway is not markedly influenced by temperature variations under the experimental conditions. In contrast, the expression of *HEY2* was significantly reduced at 33°C compared to 37°C after three days, indicating that *HEY2* is transiently regulated in response to temperature changes. However, after a week, no significant differences in *HEY2* expression were observed between the temperature conditions, suggesting that the initial response observed is transient and not maintained over time.

4 Discussion

This study investigated the influence of temperature changes on the cellular composition and functional properties of the airway epithelium, focusing on mucociliary function, cellular differentiation, and the response to pathogenic stimuli. Using primary human bronchial epithelial cells and primary nasal epithelial cells differentiated at the air-liquid interface, we investigated whether spatial differences in cellular composition within the respiratory tract are determined by the existing temperature gradient. Specifically, we explored how a shift to a lower temperature condition, 33°C instead of 37°C, affects cellular composition, functioning, and responses to pathogenic stimuli. Our results highlight the critical role of temperature in modulating cellular function and host defense responses in airway epithelial cultures.

Our analysis revealed that temperature influences the expression of genes associated with specific epithelial cell types. When temperature was switched to 33°C, the goblet cell-related genes (*MUC5AC*) in ALI-PBECs showed significantly higher expression compared to cell cultures remaining at 37°C, whereas ciliated cell-related genes (*FOXJ1*) exhibited significantly lower expression. These effects were already observable after three days of exposure to the two different temperatures, although variability among donors was noted. After one week, the effects became more pronounced, with significant differences between the two temperature conditions for both goblet and ciliated cell-associated gene. These results indicate that prolonged exposure to different temperatures amplifies its effect on cellular differentiation in bronchial epithelial cells. In contrast, club cell-related genes (*SCGB1A1*) did not show consistent temperature-dependent changes, suggesting a pronounced effect by temperature on specific airway epithelial cells and on not overall differentiation. The observed increase in the goblet cell-related gene at 33°C could be attributed to a compensatory upregulation of mucus production at lower temperatures, as previously suggested [14, 15]. However, this hypothesis requires further validation in our samples, which can be achieved through Western blot or ELISA. This finding may partially explain the higher prevalence of goblet cells in proximal airways, which are typically exposed to cooler air. Conversely, the reduced expression of the ciliated cell-related gene at 33°C highlights the temperature sensitivity of differentiation and function of these cells. These changes in cellular composition could significantly contribute to airway epithelial remodeling, a pathological process frequently observed in chronic diseases such as asthma and COPD, where altered proportions of goblet and ciliated cells compromise airway function. Interestingly, these patterns were less pronounced in nasal epithelial cells compared to bronchial epithelial cells, albeit tested in only one donor. This discrepancy may reflect intrinsic differences in the regulation of cellular composition, while the tested functional responses were showing comparable trends between nasal and bronchial epithelium. Future studies should explore the molecular mechanisms underlying these regional variations to better understand the distinct roles of epithelial subpopulation regulation.

Beyond the observed effects on the expression of genes associated with specific epithelial cell types, our results demonstrate that a reduction in temperature also affects the mucociliary function (MCC) of ciliated cells. Compared to cultures main-

tained at 37°C, those switched to 33°C generally showed reduced MCC, although inter-donor variability hindered the identification of conclusive patterns. Interestingly, ciliated cells cultured at 33°C showed less organized ciliary movement and alignment compared to those at 37°C. These observations are in line with previous studies showing an increase in ciliary beat frequency (CBF) with increasing temperature [10, 11]. A possible explanation for the inconsistent visibility of ciliary movement and the observed inter-donor variability could be attributed to the differentiation period of primary bronchial epithelial cells at the air-liquid interface, which was limited to three weeks. Several studies have reported that cilia of bronchial epithelial cells begin to differentiate within a relatively short time, typically observable as early as around ten days [19, 20]. However, it is established that the complete maturation of ciliated cells, essential for optimal mucociliary function, generally requires four to six weeks [21]. Therefore, to obtain a more accurate quantification of mucociliary function and ciliary alignment, it would be valuable to extend the culture period to allow the complete maturation of the ciliated cells. In addition, further investigation and quantitative analysis, using a MATLAB script, could provide precise measurements, offering a more detailed understanding of the key parameters influencing mucociliary clearance in this context.

Temperature variations also modulated the response of the respiratory epithelial cultures to pathogenic stimuli, such as Poly(I:C) and insults such as whole cigarette smoke (WCS). Bronchial and nasal epithelial cell cultures maintained at 33°C exhibited an enhanced antiviral response, as evidenced by increased IFNL expression after Poly(I:C) treatment. This response may suggest a functional adaptation to lower temperatures, wherein the epithelium compensates for potentially reduced mucociliary clearance or regions with elevated viral exposure by enhancing sensitivity to viral stimuli, likely through molecular pathways associated with interferon signaling. Moreover, the increased *IFNL* expression at 33°C compared to 37°C indicates that lower temperatures may amplify specific host defense pathways, enhancing the epithelium's ability to respond to viral threats. These findings are inconsistent with previous studies showing that temperature influences the antiviral activity of epithelial cells. For instance, one study observed that higher temperatures reduce viral replication in epithelial models, attributing this effect to enhanced interferon responses at higher temperatures [22]. Similarly, a study on primary human bronchial and nasal cells reported that physiological temperatures enhance the production of inflammatory cytokines, whereas exposure to lower temperatures impairs interferon-induced antiviral responses in airway epithelial cells [23]. This suggests that further research is needed with actual virus infections to further study the obtained results.

Exposure of bronchial epithelial cell cultures to whole cigarette smoke (WCS) resulted in increased *CXCL8* expression at both temperature conditions, with no significant difference between 33°C and 37°C. This suggests that temperature variation does not modulate the acute inflammatory response of bronchial epithelial cells to smoke. However, in nasal epithelial cells, a reduction in *CXCL8* expression was observed at 33°C compared to 37°C after 24 hours. This reduction is specifically attributable to the lower temperature, as transitioning the cultures after WCS exposure back from 33°C to 37°C restores the cellular response to a level comparable to that observed at 37°C. This may indicate that nasal epithelial cells are less sensitive to the pro-inflammatory effects of cigarette smoke at lower temperatures, potentially indicating a differential capacity to respond to oxidative stress-induced damage. Additionally, the decreased expression of the *HMOX1* gene in nasal cells at 33°C compared to 37°C in response to WCS highlights a thermal modulation of the oxidative stress response pathway. However, it should be noted that these results are based on nasal epithelial cells derived from a single donor, which may influence the generalizability of the observations. These findings are consistent with the results reported in [24], which indicate a reduced production of pro-inflammatory cytokines at lower temperatures in response to oxidative stimuli. This suggests that temperature not only regulates the immune and inflammatory responses of nasal epithelial cells but also modulates cellular stress response pathways, with distinct effects between viral and oxidative stimuli. An important observation from the data is that the lack of response of nasal epithelial cells upon temperature shift from 33°C to 37°C does not appear to be exclusively attributable to the effects of incubation temperature. Rather, the 33°C $\rightarrow$ 37°C control indicates that at

33°C, the oxidative stress response is not properly activated, suggesting a functional impairment of the oxidative stress pathway at lower temperatures. This observation contrasts with results obtained in bronchial cells, where the response was restored in most donors after switching to 37°C. The lack of activation at 33°C may reflect an intrinsic sensitivity of nasal cells to low temperatures or a specific disruption in the signaling mechanisms regulating *HMOX1* expression. Nevertheless, the use of cells from a single donor underscores the need for future studies involving a larger cohort of donors to confirm these findings. Additionally, further research should investigate the molecular mechanisms underlying these differences and evaluate how temperature modulation could be used to optimize therapeutic strategies.

The Notch signaling pathway is well-known for its role in regulating cellular differentiation and playing a critical role in ciliated cell versus goblet cell differentiation [18]. In the current study, we expected that exposure to lower temperatures would activate this pathway, given the predominant effects observed on these two cell types. However, our results did not show any statistical differences in the expression of the NOTCH target genes *HES1* and *HEY1* between the two temperature conditions, either after three days or one week after the temperature change. Additionally, we observed a transient reduction in *HEY2* expression, a key Notch target, at 33°C where we would have expected an increase. One possible explanation could involve interactions between Notch and other regulatory pathways. For instance, studies have shown that thermal stress can activate alternative pathways, such as NF-kB and HIF-1α, which may compete with Notch for molecular resources or negatively influence its activity [25]. Furthermore, the reduction in *HEY2* expression might reflect a negative feedback mechanism aimed at preventing earlier overactivation of the pathway. In summary, our findings highlight the complexity of Notch pathway regulation in response to temperature and suggest that further studies are needed to clarify the temporal dynamics and interactions with other molecular pathways. A better understanding of these mechanisms could open new avenues for therapeutic interventions targeting Notch or other signaling pathway modulation in respiratory diseases.

Despite the promising findings, the current study has several limitations. First, six primary bronchial epithelial cell donors and only a single donor for primary nasal epithelial cells were included, making direct comparisons between the two airway epithelial cell types challenging. Additionally, the observed inter-donor variability highlights the need to include a larger number of donors to account for individual differences and improve the accuracy of the results. Another limitation is the focus on in vitro models. While these models allow for a controlled study of specific factors, they may reduce the generalizability of findings to in vivo conditions, where mechanical factors, such as airflow also play a critical role in modulating cellular responses. In this study, primary bronchial epithelial cells were differentiated under air-liquid interface conditions for three weeks before being exposed to two different temperatures for periods of three and seven days. However, as previously discussed, the full maturation of ciliated cells in bronchial epithelial cultures typically occurs after four to six weeks. Therefore, future studies should examine the effects of temperature variations on mucociliary function and ciliary beat frequency after complete cellular maturation.

5 Conclusion

In conclusion, our findings demonstrate that temperature variations significantly influence cellular composition, change mucociliary functioning, and host defense responses of the respiratory epithelium. These temperature-dependent effects have important implications for understanding airway remodeling in respiratory diseases. Further research is needed to validate these findings in vivo and to explore the clinical applicability of temperature-targeted interventions in the management of airway diseases.

References

[1] J. D. Davis and T. P. Wypych, "Cellular and functional heterogeneity of the airway epithelium," *Mucosal immunology*, vol. 14, no. 5, pp. 978–990, 2021.

[2] P. S. Hiemstra, P. B. McCray Jr, and R. Bals, "The innate immune function of airway epithelial cells in inflammatory lung disease," *European respiratory journal*, vol. 45, no. 4, pp. 1150–1162, 2015.

[3] C. H. Dean and R. J. Snelgrove, "New rules for club development: new insights into human small airway epithelial club cell ontogeny and function," pp. 1355–1356, 2018.

[4] Y. Fang, H. Liu, H. Huang, H. Li, A. Saqi, L. Qiang, and J. Que, "Distinct stem/progenitor cells proliferate to regenerate the trachea, intrapulmonary airways and alveoli in covid-19 patients," *Cell research*, vol. 30, no. 8, pp. 705–707, 2020.

[5] M. C. Basil, J. Katzen, A. E. Engler, M. Guo, M. J. Herriges, J. J. Kathiriya, R. Windmueller, A. B. Ysasi, W. J. Zacharias, H. A. Chapman, *et al.*, "The cellular and physiological basis for lung repair and regeneration: past, present, and future," *Cell stem cell*, vol. 26, no. 4, pp. 482–502, 2020.

[6] A. Yaghi and M. B. Dolovich, "Airway epithelial cell cilia and obstructive lung disease," *Cells*, vol. 5, no. 4, p. 40, 2016.

[7] M. Khosravi, R.-L. Lin, A. P. Maskey, S. Pandey, A.-H. Lin, and L.-Y. Lee, "A distinct difference between air and mucosal temperatures in human respiratory tract," *Frontiers in Medicine*, vol. 8, p. 650637, 2021.

[8] W. Nikolaizik, J. Hahn, M. Bauck, and S. Weber, "Comparison of ciliary beat frequencies at different temperatures in young adults," *ERJ Open Research*, vol. 6, no. 4, 2020.

[9] E. McFadden Jr, B. Pichurko, H. F. Bowman, E. Ingenito, S. Burns, N. Dowling, and J. Solway, "Thermal mapping of the airways in humans," *Journal of applied physiology*, vol. 58, no. 2, pp. 564–570, 1985.

[10] C. Smith, R. Hirst, M. Bankart, D. Jones, A. Easton, P. Andrew, and C. O'Callaghan, "Cooling of cilia allows functional analysis of the beat pattern for diagnostic testing," *Chest*, vol. 140, pp. 186–90, 12 2010.

[11] M. Jorissen and A. Bessems, "Influence of culture duration and ciliogenesis on the relationship between ciliary beat frequency and temperature in nasal epithelial cells," *European archives of oto-rhino-laryngology*, vol. 252, pp. 451–454, 1995.

[12] L. Deng, P. Ma, Y. Wu, Y. Ma, X. Yang, Y. Li, and Q. Deng, "High and low temperatures aggravate airway inflammation of asthma: evidence in a mouse model," *Environmental Pollution*, vol. 256, p. 113433, 2020.

[13] A. Han, S. Deng, J. Yu, Y. Zhang, B. Jalaludin, and C. Huang, "Asthma triggered by extreme temperatures: from epidemiological evidence to biological plausibility," *Environmental Research*, vol. 216, p. 114489, 2023.

[14] M. Li, Q. Li, G. Yang, V. P. Kolosov, J. M. Perelman, and X. D. Zhou, "Cold temperature induces mucin hypersecretion from normal human bronchial epithelial cells in vitro through a transient receptor potential melastatin 8 (trpm8)–mediated mechanism," *Journal of Allergy and Clinical Immunology*, vol. 128, no. 3, pp. 626–634, 2011.

[15] G. G. Giesbrecht, "The respiratory system in a cold environment." *Aviation, space, and environmental medicine*, vol. 66, no. 9, pp. 890–902, 1995.

[16] D. K. Ninaber, A. M. van der Does, and P. S. Hiemstra, "Isolating bronchial epithelial cells from resected lung tissue for biobanking and establishing well-differentiated air-liquid interface cultures," *JoVE (Journal of Visualized Experiments)*, no. 195, p. e65102, 2023.

[17] G. D. Amatngalim, J. A. Schrumpf, F. Dishchekenian, T. C. Mertens, D. K. Ninaber, A. C. van der Linden, C. Pilette, C. Taube, P. S. Hiemstra, and A. M. van der Does, "Aberrant epithelial differentiation by cigarette smoke dysregulates respiratory host defence," *European Respiratory Journal*, vol. 51, no. 4, 2018.

[18] G. Cumplido-Laso, D. A. Benitez, S. Mulero-Navarro, and J. M. Carvajal-Gonzalez, "Transcriptional regulation of airway epithelial cell differentiation: insights into the notch pathway and beyond," *International Journal of Molecular Sciences*, vol. 24, no. 19, p. 14789, 2023.

[19] Z. Bukowy-Bieryłło, P. Daca-Roszak, J. Jurczak, H. Przystałowska-Macioła, R. Jaksik,

M. Witt, and E. Zietkiewicz, "In vitro differentiation of ciliated cells in ali-cultured human airway epithelium–the framework for functional studies on airway differentiation in ciliopathies," *European Journal of Cell Biology*, vol. 101, no. 1, p. 151189, 2022.

[20] P. M. de Jong, M. A. van Sterkenburg, S. C. Hesseling, J. A. Kempenaar, A. A. Mulder, A. M. Mommaas, J. H. Dijkman, and M. Ponec, "Ciliogenesis in human bronchial epithelial cells cultured at the air-liquid interface." *American journal of respiratory cell and molecular biology*, vol. 10, no. 3, pp. 271–277, 1994.

[21] D. Gras, A. Petit, J. Charriot, L. Knabe, K. Alagha, A. S. Gamez, C. Garulli, A. Bourdin, P. Chanez, N. Molinari, *et al.*, "Epithelial ciliated beating cells essential for ex vivo ali culture growth," *BMC pulmonary medicine*, vol. 17, pp. 1–7, 2017.

[22] E. Foxman, J. Storer, M. Fitzgerald, B. Wasik, L. Hou, H. Zhao, P. Turner, A. Pyle, and A. Iwasaki, "Temperature-dependent innate defense against the common cold virus limits viral replication at warm temperature in mouse airway cells," *Proceedings of the National Academy of Sciences*, vol. 112, 01 2015.

[23] V. T. Mihaylova, Y. Kong, O. Fedorova, L. Sharma, C. S. Dela Cruz, A. M. Pyle, A. Iwasaki, and E. F. Foxman, "Regional differences in airway epithelial cells reveal tradeoff between defense against oxidative stress and defense against rhinovirus," *Cell Reports*, vol. 24, no. 11, pp. 3000–3007.e3, 2018.

[24] M. Eimonte, N. Eimantas, L. Daniuseviciute, H. Paulauskas, A. Vitkauskiene, G. Dauksaite, and M. Brazaitis, "Recovering body temperature from acute cold stress is associated with delayed proinflammatory cytokine production in vivo," *Cytokine*, vol. 143, p. 155510, 2021.

[25] Z. W. Dong, J. Chen, Y. C. Ruan, T. Zhou, Y. Chen, Y. Chen, L. L. Tsang, H. C. Chan, and Y. Z. Peng, "Cftr-regulated mapk/nf-κb signaling in pulmonary inflammation in thermal inhalation injury," *Scientific reports*, vol. 5, no. 1, p. 15946, 2015.

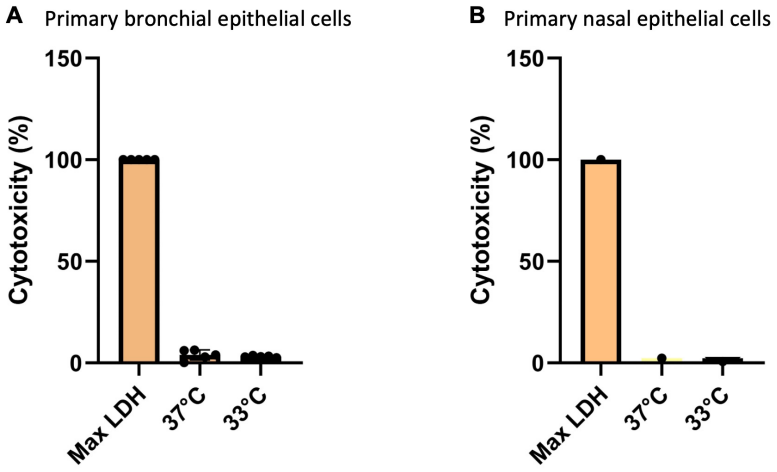
SUPPLEMENTARY MATERIALS

Supplementary Table 1: Primer sequences used for quantitative PCR

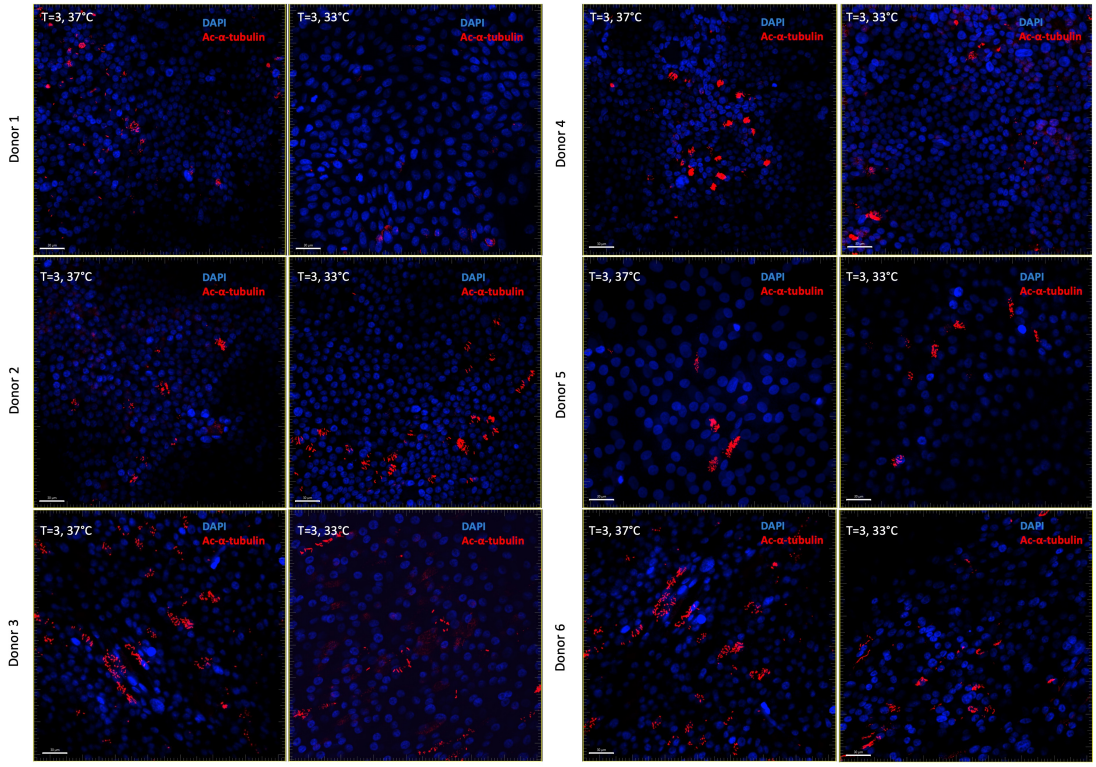
Gene	Forward sequence (5' to 3')	Reverse sequence (3' to 5')
OAZ1	GGATCCTCAATAGCCACTGC	TACAGCAGTGGAGGGAGACC
RPL22	TCGCTCACCTCCCTTTCTAA	TCACGGTGATCTTGCTCTTG
FOXJ1	GGAGGGGACGTAAATCCCTA	TTGGTCCCAGTAGTTCCAGC
SCGB1A1	ACATGAGGGAGGCAGGGGCTC	ACTCAAAGCATGGCAGCGGCA
MUC5AC	CCTTCGACGGACAGAGCTAC	TCTCGGTGACAACACGAAAG
CXCL8	CTGGACCCCAAGGAAAAC	TGGCAACCCTACAACAGAC
IFNL	GGACGCCTTGGAAGAGTCACT	AGAAGCCTCAGGTCCCAATTC
HES1	CCTGTCATCCCCGTCTACAC	CACATGGAGTCCGCCGTAA
HEY1	ATCTGCTAAGCTAGAAAAAGCCG	GTGCGCGTCAAAGTAACCT
HEY2	AAGGCGTCGGGATCGGATAA	AGAGCGTGTGCGTCAAAGTAG

Supplementary Table 2: Primary antibodies used for immunofluorescence staining

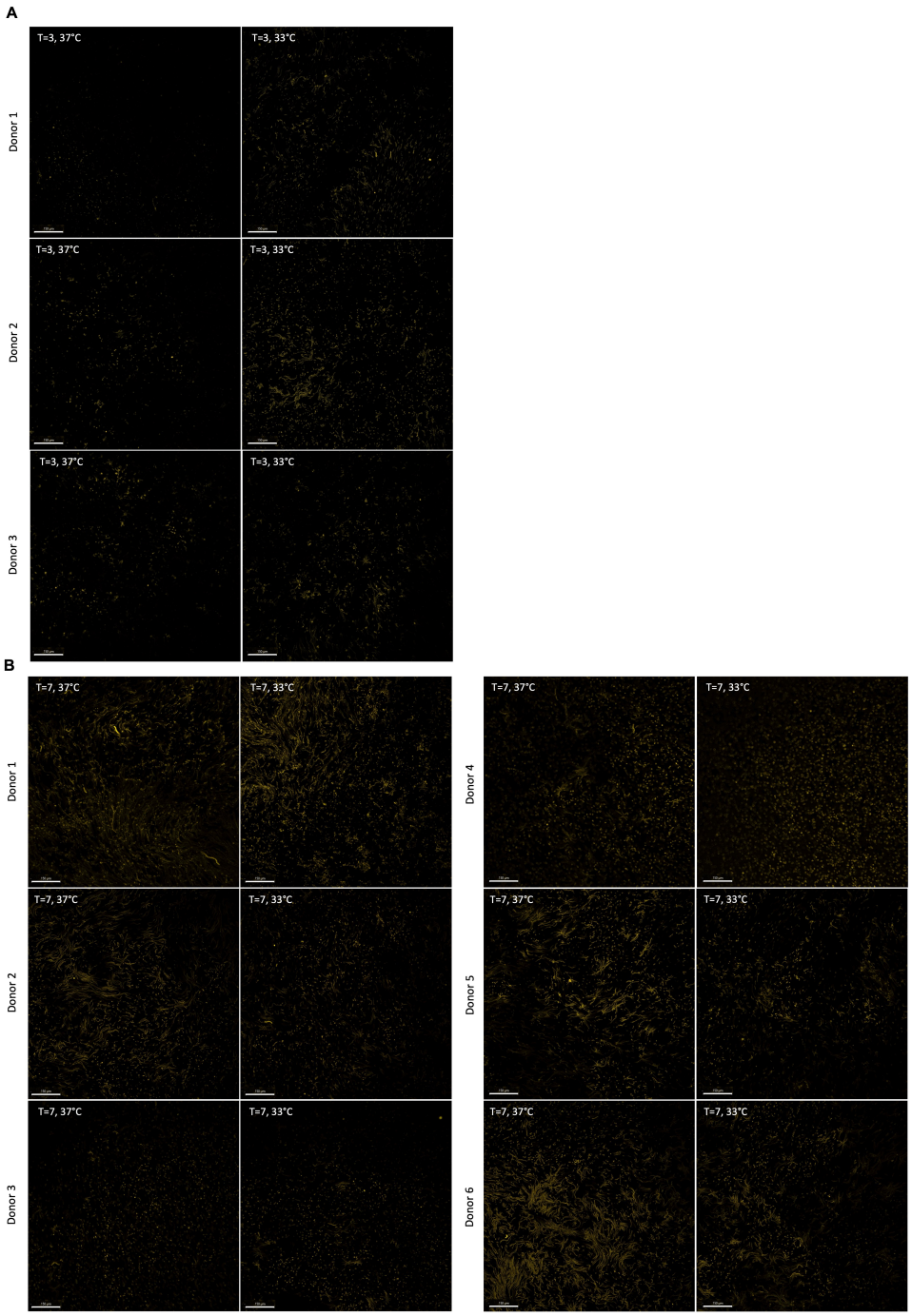
Antibody	Supplier	Catalog #	Species	Antibody dilution
Mucin 5AC	Abcam	218363	Rabbit	1:200
Acetylated α-Tubulin	Sigma Aldrich	T6793	Mouse	1:400



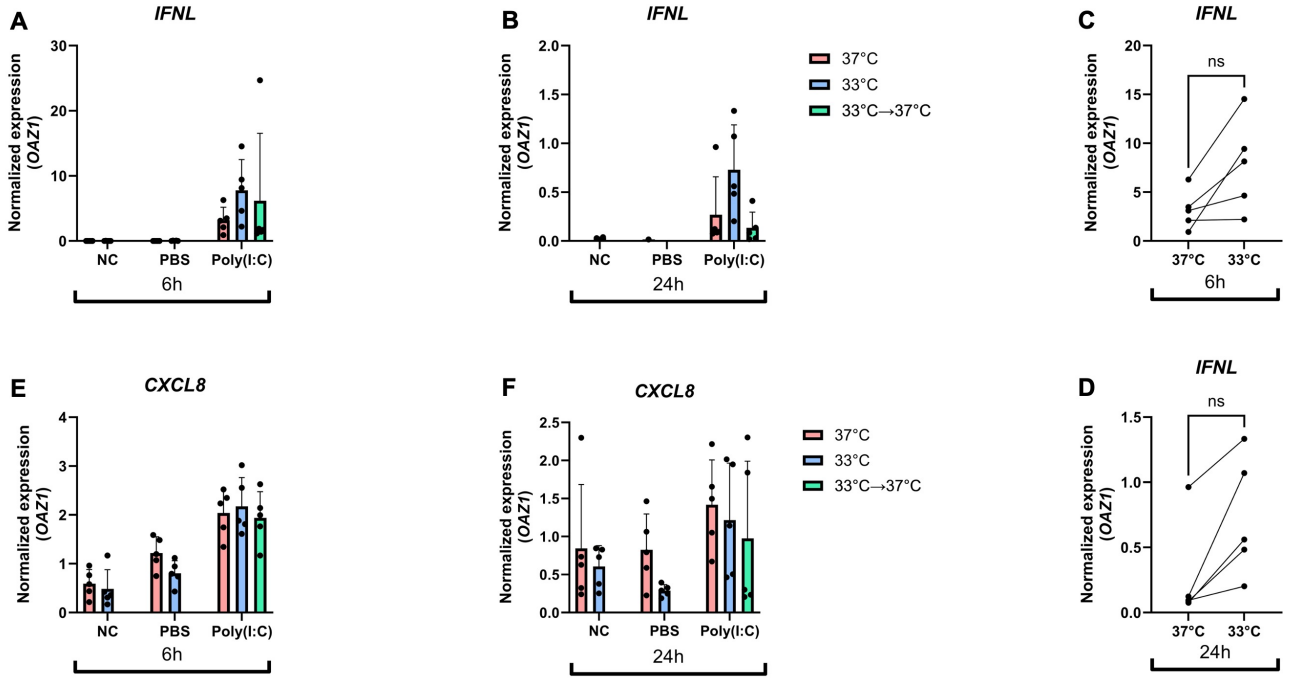
Supplementary Fig. 1. Cytotoxicity levels expressed as the percentage of lactate dehydrogenase (LDH) release in epithelial cells under three experimental conditions: positive control (Max LDH), cultures maintained at 37°C, and cultures maintained at 33°C. Cytotoxicity levels in both bronchial epithelial cells (left) and nasal epithelial cells (right) remained extremely low at both 37°C and 33°C, indicating excellent cell membrane integrity. No significant differences were observed between the two temperature conditions.



Supplementary Fig. 2. Representative immunofluorescence staining of ciliated cells in bronchial epithelial cultures using acetylated α-tubulin as a marker, three days after the temperature shift. Cultures were maintained at 37°C (left) or shifted to 33°C (right). Images illustrate the distribution of acetylated α-tubulin (red), highlighting the cilia of ciliated cells, with nuclei counterstained using DAPI (blue). Scale bar: 30 µm.



Supplementary Fig. 3. Images of fluorescent bead displacement in bronchial epithelial cell cultures from different donors. **A**. Fluorescent bead displacement on day 3 after the temperature shift. Cultures maintained at 37°C (left) and at 33°C (right). At this time point, bead displacement was barely detectable in both conditions. **B**. Fluorescent bead displacement on day 7 after the temperature shift. Cultures maintained at 37°C (left) and at 33°C (right). Bead displacement varied among donors: in some cultures, displacement increased at 33°C compared to 37°C, whereas in others, the opposite trend was observed. Scale bar: 150 μm.



Supplementary Fig. 4. Effect of temperature changes on the inflammatory gene expression in ALI-PBECs (after outlier removal). **A.** Gene expression of *IFNL* normalized to *OAZ1* after 6 hours following the temperature shift in the NC, PBS, and Poly(I:C) treatment groups at 37°C, 33°C, and 33°C $\rightarrow$ 37°C. No significant differences (ns) were observed between conditions after removing an identified outlier. N = 6 donors. **B.** Gene expression of *IFNL* normalized to OAZ1 after 24 hours following the temperature shift in the NC, PBS, and Poly(I:C) treatment groups at 37°C, 33°C, and 33°C $\rightarrow$ 37°C. No significant differences were observed after outlier removal. N = 6 donors. **C.** Paired *IFNL* expression at 37°C and 33°C after 6 hours following the temperature shift, normalized to *OAZ1*. No significant differences were observed using the Wilcoxon matched-pairs signed rank test after removing an outlier. N = 6 donors. **D.** Paired *IFNL* expression at 37°C and 33°C after 24 hours following the temperature shift, normalized to *OAZ1*. No significant differences were observed using the Wilcoxon matched-pairs signed rank test after removing an outlier. N = 6 donors. **E.** Gene expression of *CXCL8* normalized to *OAZ1* after 6 hours following the temperature shift in the NC, PBS, and Poly(I:C) treatment groups at 37°C, 33°C, and 33°C $\rightarrow$ 37°C. No significant differences were observed after removing the same outlier identified in the *IFNL* analysis. N = 6 donors. **F.** Gene expression of *CXCL8* normalized to *OAZ1* after 24 hours following the temperature shift in the NC, PBS, and Poly(I:C) treatment groups at 37°C, 33°C, and 33°C $\rightarrow$ 37°C. No significant differences were observed after removing the same outlier identified in the *IFNL* analysis. N = 6 donors.

Literature review

Contribution of various factors to spatial patterns in airway epithelial composition

Elena Ferraris[1], Massimo Mastrangeli[2], Anne M. van der Does[1]
[1]PulmoScience Lab, Department of Pulmonology, Leiden University Medical Center, Leiden
[2]Electronic Components, Technology and Materials, Department of Microelectronics, Delft University of Technology, Delft
04-09-2024

Abstract

The airway epithelium plays a crucial role in maintaining homeostasis by regulating fluid balance, mucus production, and protection against inhaled particles. Throughout the respiratory tree, the epithelial cell composition undergoes continuous changes, resulting in distinct spatial patterns. Chronic lung diseases, such as COPD and asthma, involve significant remodeling of the airway epithelium, characterized by alterations in cell composition and function, which distort these spatial patterns. Understanding the regulation of these spatial patterns under homeostatic conditions could provide therapeutic opportunities to address epithelial remodeling in chronic lung diseases. However, there is limited knowledge about the factors and mechanisms that contribute to these patterns. This study aims to provide an in-depth overview of factors that regulate spatial patterns in the cellular composition of the airway epithelium under homeostatic conditions. A literature review was conducted, identifying key elements such as temperature, mechanical forces, pH, humidity, and microbiome that form gradients along the airway tree. Results indicate that temperature changes influence the ciliary beat frequency, while mechanical forces such as stretching and compression modulate goblet cell density and mucus production. Specifically, stretching tends to reduce the number of goblet cells, whereas compression increases mucus secretion. Additionally, variations in pH, and humidity alter mucus viscosity and ciliary activity, impacting the epithelial composition. Despite the need for additional studies, these findings suggest a dynamic interplay between these factors and the epithelium, contributing to the spatial organization of airway epithelial cells. Understanding these mechanisms is essential for identifying therapeutic targets for airway remodeling in chronic lung diseases.

Introduction

The airway epithelium is a complex cell layer of specialized cells that forms the interface between the outside environment and the tissue underneath and interacts with factors locally present on both sides, including microbes, and the immune system. It performs several essential functions for maintaining homeostasis, including fluid balance, mucus production for mucociliary clearance, barrier protection, elimination of harmful inhaled particles, and activation of inflammatory cells in response to injury (Davis & Wypych, 2021). The respiratory epithelium consists of numerous cell types and subsets, which are both morphologically and functionally different (Fig.1). The key players include basal cells, ciliated cells, secretory cells (goblet and club), and neuroendocrine cells (Hiemstra et al., 2015). The properties and prevalence of each cell type differ along the proximal-distal respiratory axis. For example, basal cells are distributed throughout the entire airway epithelium, but their prevalence gradually decreases in the distal airways (Dean & Snelgrove, 2018). Basal cells are progenitor cells that play an important role in the maintenance, regeneration, and repair of the epithelium

(Fang et al., 2020). Ciliated cells are the predominant cell type at the tracheobronchial level and are responsible for promoting mucus transport through the coordinated movement of their apical cilia (Yaghi & Dolovich, 2016). Goblet cells, however, are rich in mucin-filled granules, which are released to capture pathogens in the airways and are higher in number in the proximal airways compared to the distal airways (Davis & Wypych, 2021). In contrast, the epithelium of the distal airways contains a higher number of club cells, which partially replace the functions of basal and goblet cells. These club cells are crucial for the protection and maintenance of the airways. Furthermore, both basal and club cells act as progenitor cells, differentiating into specialized cell types (Rokicki et al., 2016). Finally, the respiratory epithelial environment also contains non-epithelial cells such as stromal cells, including fibroblasts and immune cells nearby (Davis & Wypych, 2021).

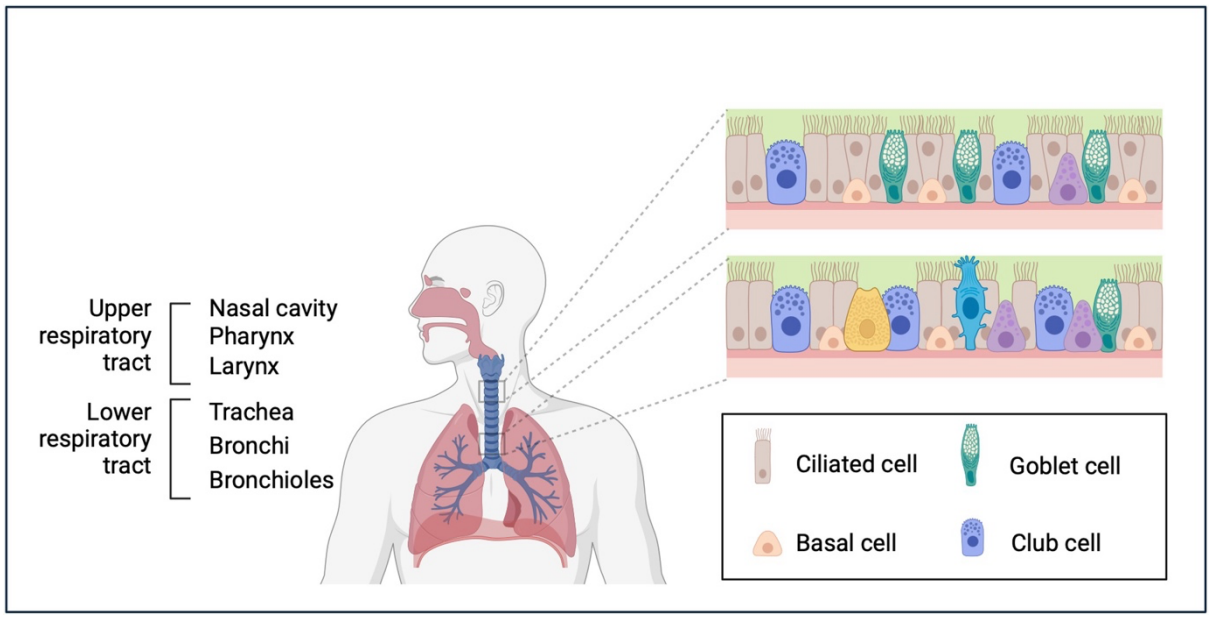


Fig. 1. Representation of the main cells and their distribution along the respiratory tract. Not all cells are shown; instead, only the key players are highlighted, including ciliated cells, club cells, basal cells, and goblet cells. Created with BioRender.

Continuous exposure to airborne pathogens, air pollution, and tobacco smoke promotes chronic immune responses, inflammation, and repair processes leading to damage to the airways (Dean & Snelgrove, 2018). Damage to the airways can result in substantial structural changes and remodeling of the airway epithelium. Airway epithelial remodeling is characterized by a change in cellular composition, driving various pathologies and a prominent factor in several chronic lung diseases, including chronic obstructive pulmonary disease (COPD), and asthma (Jeffery, 2001). Several studies investigated airway epithelial changes in patients with chronic lung diseases. Shaykhiev and Crystal (2014) showed alterations in the small airways which affected the airway wall, the airway epithelium, and small bronchioles. These alterations included airway epithelial remodeling with basal cell and mucosal cell hyperplasia, squamous metaplasia, altered ciliated cell differentiation, loss of club cells, and reduced apical junctional barrier integrity. Raby et al. (2023) reported an increase in basal cells in the airways of patients with COPD. These cells show a compromised capacity for both regeneration and differentiation, leading to an accumulation of dysfunctional cells. Metaplasia and hyperplasia of goblet cells are related to a deregulation of the expression of transcription factors such as NOTCH1 and HEY2, which contribute to the increase in goblet cell formation

and related mucus secretion at the cost of ciliated cells thereby further damaging the mucociliary machine (Ganesan & Sajjan, 2013). Additionally, a reduction in the length of cilia and a decrease in the club cells in the small airway epithelium was observed. This reduction causes the composition of epithelial cells in the small, distal airways to shift towards a pattern that more closely resembles that of the larger, proximal airways (Raby et al., 2023). Also, Yang et al. (2017) demonstrated that the arrangement of cells along the proximal-distal axis is altered in COPD patients. As a result of elevated EGF/EGFR signaling, smaller airway cells lose their phenotype and gain the phenotype of the proximal airway epithelium. EGFR is expressed in airway epithelial cells and can be activated when EGF or some of its other ligands bind to EGFR. Activation of EGFR triggers a cascade of intracellular signals involving several signal transduction pathways, depending on the ligand involved. Elevated EGFR signaling in the distal airway cells promotes a phenotypic change, causing the small airway cells to lose their features and acquire the properties of the proximal airway epithelium cells (i.e. increase in goblet cell number) (Raby et al., 2023 & Yang et al.,2017). Structural and functional airway alterations are also visible in patients with asthma. The main aspects of remodeling are the thickening of the basement membrane, airway fibrosis, and goblet cell hyperplasia. Hyperplasia of goblet cells leads to an increase in mucus-producing cells, leading to hypersecretion and airway obstruction via mucus plugging (Varricchi et al., 2024). Moreover, the reduction in ciliated cells further diminishes the mucociliary clearance capacity of the epithelium, thereby impairing the efficient removal of excess mucus and inhaled particles (Raby et al., 2023). Airway epithelial cells of asthma patients are subjected to increased mechanical forces, such as compression, stretching, and stress as a result (Wirtz & Dobbs, 2000). An increase in mechanical stimulation is one of the causes of the overproduction of mucus, fibronectin, and collagen III and V (Varricchi et al., 2024). Furthermore, a reduction in the number of club cells and a decrease in their regenerative capacity and production of protective proteins such as secretoglobin 1A1 (SCGB1A1) is observed (Varricchi et al., 2024 & Raby et al., 2023). These findings indicate that multiple signaling pathways are involved in airway remodeling processes, demonstrating the complexity of structural and functional alterations in chronic lung diseases.

Understanding the cellular and molecular mechanisms that regulate the structure and function of the airway epithelium throughout the respiratory tree could facilitate the identification of therapeutic opportunities for addressing airway remodeling, a hallmark of several chronic lung diseases. Although many studies examined structural and functional changes in patients with chronic lung diseases, the mechanisms that specifically determine the spatial patterns in the cellular composition of the airway epithelium in healthy individuals remain unknown. Understanding these mechanisms is highly relevant, as the factors that control these spatial patterns also regulate the cellular composition of the airway epithelium. Therefore, identifying factors that can controllably promote or inhibit the differentiation of basal cells into specialized cells and determine the cellular balance for example between goblet cell/ciliated cell ratio could reveal new therapeutic targets for the treatment of conditions involving epithelial remodeling.

The objective of this study is to investigate the scientific evidence for factors that regulate and influence spatial patterns in the cellular composition of the airway epithelium under homeostatic conditions. We hypothesize that the variations in the properties of these factors along the airway tree may induce distinct structural and functional changes in the airway epithelium. To achieve this objective, we will first investigate when spatial patterns occur

during the development of the airway epithelium and how these patterns evolve. Secondly, we aim to identify the factors that establish a similar gradient along the airway tree and analyze if and how these factors influence spatial patterns in the composition of airway epithelial cells.

Development of the airway epithelium

The airway epithelium undergoes significant changes during fetal development and after birth contributing to the spatial differences in epithelial cell composition observed in adult lungs. These changes are essential for the adaptation of the respiratory system to extrauterine life and for the protection of airways from pathogens and inhaled particles (Nino et al., 2022). It is important to note that studies performed in animals need to be interpreted with care as mice do not display a matched gradient in cellular composition along the respiratory tree as humans (Danopoulos et al., 2019). Spatial variations in epithelial cell composition differ between species. Despite these differences, valuable insights can still be gained from existing knowledge (Eenjes et al., 2022).

Toskala et al. (2005) demonstrated how the temporal and spatial patterns of the tracheobronchial airways evolve during postnatal development. Their study indicated that the development of ciliated cells in the mouse airway follows a specific temporal sequence, with initial progression at the tracheobronchial level starting from day 16 of gestational age. Subsequently, the presence of ciliated cells in the distal airway epithelium becomes visible from day 18, and a significant increase in cell density across the proximal distal tract is observed from day 19 (Toskala et al.,2005). After birth, the density of ciliated cells in the proximal airways of mice increases significantly by the first postnatal day and continues to increase linearly until the 21st postnatal day (Francis et al., 2009). In contrast, the number of ciliated cells in the terminal bronchioles of mice increases only after the first postnatal week and remains lower than that in the more proximal airways. The differences in ciliated cell density between the proximal and distal areas remain significant even after achieving typical adult density (Toskala et al.,2005).
Differentiation and maturation of the human airway epithelium occur in a gradient from the proximal to the distal tract, similar to observations in mouse models (Eenjes et al., 2022). Gaillard et al. (1989) reported that the differentiation of ciliated cells in the human respiratory epithelium begins around the 20th to 23rd week of gestation. In contrast, subsequent studies indicated that such differentiation occurs at the end of the pseudoglandular phase, between the 6th and 16th weeks of gestation (Jeffrey, 1998). During the early stage, ciliated cells are randomly arranged and have relatively short cilia. Significant elongation of the cilia and proper orientation of ciliated cells are observed only at the end of the canalicular period (Gaillard et al., 1989 & Jeffery, 1998). The differentiation of secretory cells, which include goblet cells and club cells, begins at the end of the pseudoglandular period, concurrent with the differentiation of the ciliated cells (Jeffery, 1998). Goblet cells, which are responsible for mucus production, begin to mature from week 13 of gestation, while club cell maturation is around week 19, following the induction of the SCGB1A1 marker (Jeffery, 1998 & Zemke et al., 2009). Both secretory cell types continue to differentiate and mature until the postnatal period (Jeffery, 1998). The number of goblet cells increases until midgestation, followed by a slight decrease and concomitant increase in ciliated cells, ensuring an ideal balance at the end of gestation (Gaillard et al., 1989 & Jeffery, 1998). Throughout the gestational period, goblet cells are more prevalent in the proximal airway epithelium compared to the distal tract. Importantly, in the

postnatal period, when the fetus starts breathing, there is an increase in the ratio of mucus-producing cells to ciliated cells, which maintains a specific spatial pattern (Jeffery, 1998).

Several studies identified signaling factors involved in goblet cell differentiation during the prenatal and postnatal periods. According to Rajavelu et al. (2015), FOXA3 is a transcription factor that promotes the proliferation of mucus-producing cells in the airway epithelium of a newborn mouse, by inducing the expression of SPDEF, MUC5B, and MUC5AC. Deactivation of Notch signaling leads to an overproduction of goblet cells in the murine airway epithelium, which is associated with increased expression of the transcription factors FOXA3, SPDEF, and MUC5AC (Tsao et al., 2011). Moreover, there is a reduction in the expression of FOXA2, a transcription factor that inhibits the differentiation and maturation of goblet cells. Inactivation of Notch signaling also results in a decrease in club cells and an increase in ciliated cells (Tsao et al., 2011). Whitsett (2018) observed that Notch signal deactivation promotes the differentiation of basal cells and club cells into ciliated cells. Studies in mice further demonstrated a correlation between club cells and goblet cells during the postnatal period. Inactivation of the protein O-fucosyltransferase 1 (Pofut1), disrupts Notch signaling, leading to the conversion of club cells into goblet cells, particularly in the proximal airway tract (Tsao et al., 2011). Thus, Notch signaling plays a critical role in maintaining club cells in their original form and function, preventing their differentiation into mucus-producing goblet cells (Tsao et al., 2009). This mechanism is essential for maintaining the balance among the various cell types in the airway epithelium. Other important factors in the regulation of airway epithelium development include β-Catenin, SOX2, SOX9, and the WNT and BMP signaling pathways (Aros et al., 2021 & Danopoulos et al., 2018).

In conclusion, the development and maturation of the airway epithelium represent a complex and highly regulated process that is essential for establishing a functional epithelium capable of protecting against pathogens and inhaled particles. Studies have highlighted a specific temporal pattern for the differentiation of ciliated, goblet, and club cells, with significant variations between proximal and distal airway tract regions and the involvement of multiple signaling pathways.

Factors that form a gradient across the airway tree

The epithelial gradient along the airway tree may be influenced by one or multiple factors that form similar gradients (Fig.2). Studies demonstrated that both temperature and mechanical exposures form a specific gradient along the airway tree. From inhalation up to gas exchange, the temperature gradually increases along the airway tree, reaching approximately 35.7 ± 0.2°C in the extrathoracic trachea and 36.9 ± 0.2°C in the segmental bronchus (Khosravi et al., 2021). The mechanical properties that vary along the airway tree include airflow resistance, mechanical shear stress, and elasticity, which refers to the ability to stretch and compress (LaPrad et al., 2013). Shear stress is generally higher in smaller airways compared to larger airways due to their reduced diameter and the increased concentration of frictional forces on a smaller surface area (Xia et al., 2010). Furthermore, the levels of elongation and compression are greater in the distal airways compared to the proximal airways due to their higher elasticity (LaPrad et al., 2013). Additionally, studies indicated that the pH of the airway surface fluid progressively increases along the tracheobronchial tract due to variations in cellular composition, the presence of mucous-secreting glands, and the distribution of ion channels (Zajac et al., 2021). Humidity and the respiratory microbiota also form gradients

along the airway epithelium (Man et al., 2017 & West, 1978). Investigating these gradients is essential for understanding how they affect spatial patterns in the composition of airway epithelial cells.

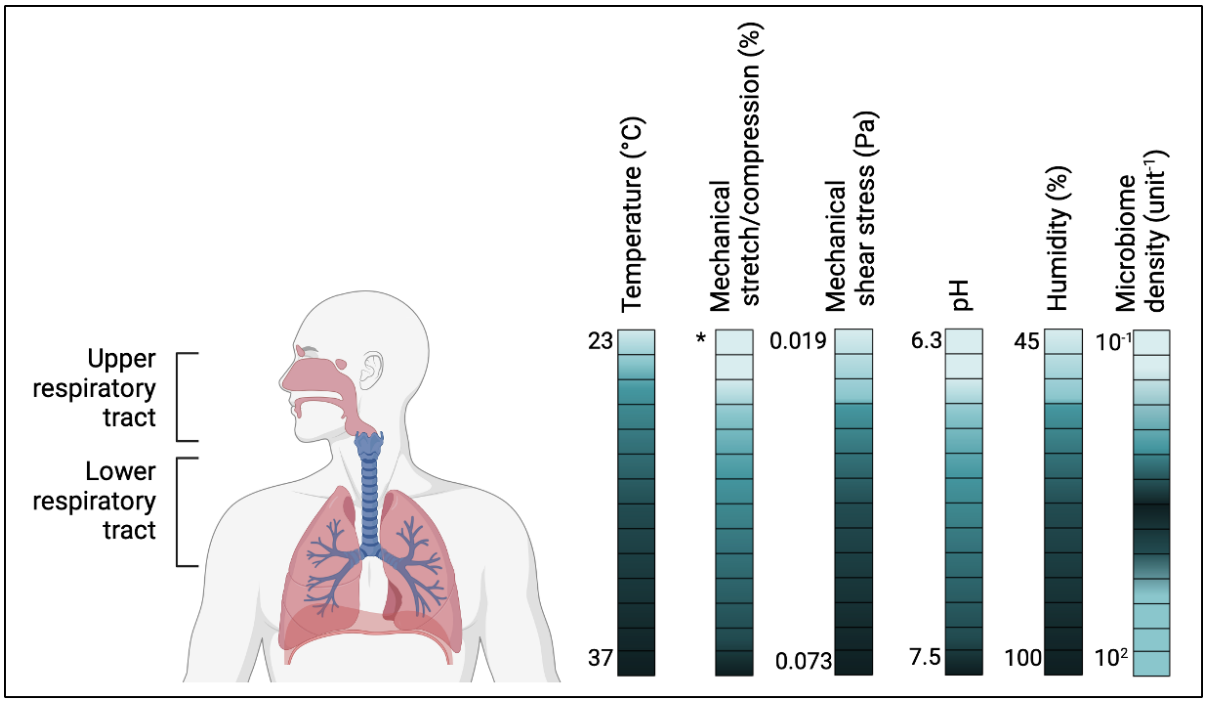


Fig. 2. Factors that form a gradient across the airway tree. Six parameters are shown using a color scale representing gradients of temperature, mechanical stretch/compression, shear stress, pH, humidity, and microbiome density (Man et al., 2017, Xia et al., 2010). An increase in color intensity corresponds to a higher measurement unit. For all parameters, a higher intensity is observed in smaller airways compared to larger airways. In contrast, microbiome density exhibits higher intensity in the central region of the airway epithelium. No specific values are given for the percentage of stretching and compression, as no relevant articles are found. However, several studies showed that larger airways, such as the trachea, are stiffer and less flexible than smaller airways, resulting in a lower capacity for stretch and compression (LaPrad et al., 2013). Created with BioRender.

How factors affect spatial patterns of airway epithelial cells

Temperature

Temperature levels can influence the differentiation and maturation of airway epithelial cells. Several studies demonstrated that temperature plays a central role in the regulation of ciliated cell motility in the epithelial airways. The frequency of ciliary beating in the nasal and tracheal mucosa exhibits an exponential response to temperature changes within the range of 9°C to 20°C (Clary-Meinesz et al., 1992). Jorissen and Bessems (1995) observed a linear increase in ciliary beat frequency within the temperature range of 20°C to 35°C. This linear trend is further confirmed by Smith et al. (2010), who demonstrated a linear change in ciliary beat frequency between 2°C and 37°C. More recently, Nikolaizik et al. (2020) confirmed the linear relationship between temperature and ciliary beat frequency, reporting frequencies of 7.0 Hz, 7.6 Hz, and 8 Hz at 25°C, 32°C, and 37°C, respectively. However, Roth et al. (2023) observed that the ciliary beat frequency in human tissue at room temperature is approximately 2.9 ± 0.5 Hz. This discrepancy from previous studies may be attributed to variations in the samples

used (e.g. tissue versus cell culture) and the experimental conditions. Finally, temperatures above 40°C significantly reduce the ciliary beat frequency, potentially making it almost non-existent and causing permanent damage to the ciliated cells (Jorissen & Bessems, 1995). When in vitro techniques were used to examine the airway mucociliary system at temperatures ranging from 40°C to 50°C for 20, 45, and 165 minutes, multiple stimulations with hot air demonstrated several morphological changes, including a decrease in ciliary length, an altered orientation of the cilia, fusion of ciliary tips, vesicle formation within the cilia, and complete deterioration of the ciliated cells after 165 minutes (Mecklenburg et al., 1974). Furthermore, exposure to high temperatures results in altered mucus production, increased airway inflammation, activation of TRP receptors (including TRPV1 and TRPV4), and activation of heat shock proteins (Deng et al., 2020 & Han et al., 2023).

Continuous exposure to low temperatures also induced morphological changes in airway epithelial cells (Li et al., 2011). Similar to the effects observed with hot air stimulation, ultrastructural alterations were present in ciliated cells (Li et al., 2011). These alterations include changes in the ciliary membrane, the presence of sub-membranous vesicles, and modifications in the architecture of ciliated cells (D'Amato et al., 2018 & Li et al., 2013). Several studies demonstrated that cold air increases the number of goblet cells, leading to enhanced mucus production in the airways (Giesbrecht, 1995). This is an interesting observation that needs further confirmation, as the larger airway, which is exposed to colder temperatures than the distal regions, harbors the highest number of goblet cells compared to other regions. The TRPM8 channel plays a crucial role in regulating mucus production and the secretion of Muc5ac. Activation of TRPM8 by cold triggers a cascade of events involving Ca2+, PLC, PIP2, and the protein MARCKS, resulting in mucus hypersecretion (Li et al., 2011).

In conclusion, extreme temperatures, whether high or low, induce significant morphological and functional changes in airway epithelial cells, impacting their composition and the spatial distribution of ciliated and goblet cells. Understanding these effects is essential for studying how spatial patterns in the composition of airway epithelial cells are influenced.

Mechanical forces

Mechanical forces can significantly influence and contribute to airway remodeling (Veerati et al., 2020). The interactions between mechanical forces and airway epithelial cells are of particular interest for understanding the cellular and molecular dynamics that regulate airway health. These interactions are essential for maintaining the structural integrity and functionality of the respiratory system. Furthermore, understanding these dynamics is crucial for developing novel therapeutic strategies for chronic respiratory diseases, where mechanical forces and cellular responses are frequently dysregulated (Veerati et al., 2020). From the prenatal stage, the airway epithelium is exposed to several mechanical forces that play a crucial role in the development and preparation of the lungs for extrauterine life (Kitterman, 1996). Several studies demonstrated that these forces influence the branching process of the embryonic airway epithelium. Varner et al. (2015) showed how intraluminal pressures and mechanical forces contribute to embryonic airway morphogenesis. Alteration of fluid-filled lumen pressure during the gestational period may impair airway epithelium formation. Additionally, other studies demonstrated changes in the expression of specific genes in response to mechanical stretch during lung development (Kitterman, 1996 & Nardo et al., 2000). This confirms the influence of mechanical stress on the proliferation and differentiation of airway epithelial cells during the prenatal phase. Subsequently, during the postnatal phase, the airway epithelium is strongly influenced by mechanical stimuli resulting from breathing, such as stretching, compression, and shear stress (Veerati et al., 2020).

Mechanical stretch

Several studies demonstrated that mechanical forces, such as stretching, significantly impact the development of the airway epithelium. Nawroth et al. (2023) observed that mechanical stimuli simulating respiratory movements influence the maturation, differentiation, and functioning of airway epithelial cells. In this study, an airway-on-chip device was used, in which a breathing-related stretch force (5%, 0.25 Hz) and an airflow-related shear stress force were applied to human primary bronchial epithelial cell cultures (hPBECs). A significant reduction in gene expression of the basal cell marker TP63 was observed after one week of applying stretch and airflow, indicating a potential decrease in basal cell population and consequently increased cell maturation compared to the control chip. Furthermore, a decrease in goblet cell numbers was observed, although this reduction was not statistically significant. Finally, gene expression of SCGB1A1, a key marker for club cell maturation, was higher in chips subjected to mechanical stimuli (Nawroth et al., 2023). This suggests that simulated respiratory movements promote the maturation of club cells. This finding is particularly interesting because, in smaller airways, where the levels of club cells are relatively high, the levels of elongation are also higher compared to larger airways (LaPrad et al., 2013). Lin et al. (2023) examined the behavior of airway basal cells under very high levels of stretch, showing increased gene expression levels of basal cell markers (KRT5) and a decrease in ciliated cell markers (Ac-tub+) after 48 hours of elongation (20%, 0.33 Hz). Furthermore, the results indicate that this level of mechanical stretch leads to a decrease in the expression of the cell proliferation marker MKI67 after 24 and 48 hours of exposure. Finally, with prolonged exposure to mechanical stretch, some studies demonstrated an increase in cell apoptosis and caspase activation (Lin et al.,2023). However, these effects may be related to the experimental model used, as well as the amount and frequency of the applied stress, as other studies did not find similar results (Nawroth et al., 2023). Nawroth's studies use an elongation percentage that reflects normal physiological conditions, whereas the studies conducted by Lin et al. (2023) use an elongation percentage of 20%, which is considered excessive (Gong et al., 2021). The loss of cilia and increased basal cell markers are characteristics of the development of fibrotic processes (Collin et al., 2021). In conclusion, variations in mechanical stretch can alter the arrangement of cells, their proliferation, and their function, potentially resulting in changes to the spatial pattern of the airway epithelium.

Mechanical compression

Compression also seems to influence the maturation, differentiation, and function of airway epithelial cells. According to Park and Tschumperlin (2009), secretion of Muc5ac in primary airway epithelial cell cultures increases following alterations in the mechanical environment, particularly during compression. The compression was quantified and analyzed following the application of a continuous apical-basal transcellular pressure (30 cm H_2O). The results demonstrate a significant increase in the percentage of cells positive for MUC5AC over time, with an increase evident from day 3, continuing up to day 14. Additional studies corroborated the rise in mucus production following continuous compressive stress on normal human bronchial epithelial cells (NHBE). Experimental data indicate that compression induces a significant release of endothelin (ET-1 and ET-2) and transforming growth factor-β2 (TGF-β2) after 4 and 8 hours, respectively (Tschumperlin et al., 2003). Elevated levels of TGF-β2, TGF-β1, and endothelin significantly contribute to increased mucus production (Veerati et al., 2020). Specifically, the modulation of mucus production in airway epithelial cells following

compression involves several intracellular signaling pathways, activation of transcription factors, and upregulation of cytokines (Fagan et al., 2001 & Henriksson et al., 2015 & Polikepahad et al., 2008 & Song et al., 2012). The increased mucus production following compression may appear contradictory, given that in the small airways, where both stretching and compression are prevalent, one would typically expect reduced mucus production. Excessive mucus in the small airways would lead to complete airway obstruction. This apparent contradiction could be explained by a correlation between compression and stretching. Data from Nawroth et al. (2023) suggested that stretching may cause a decrease in goblet cells, thereby reducing mucus production. A dynamic balance between compression and stretching may affect the amount of mucus produced and the density of goblet cells in the airways. This balance may vary significantly during different stages of prenatal and postnatal development. Further research is needed to confirm the relationship between these two mechanical forces.

Ciliated cells also change in response to the application of compressive stress. Lee et al. (2021) indicate that the application of pressure greater than 0.5 kPa increases ciliary beat frequency primarily due to an increase in the maximum ciliary velocity. Despite the increase in ciliary beat frequency, the studies did not observe any morphological changes in the ciliated cells. The observed increase in ciliary beat frequency may be a consequence of increased mucus production induced by compression. This increase aims to remove excess mucus from the airways, maintain effective mucociliary clearance, and prevent airway obstruction. In conclusion, evidence suggests that compressive stress affects spatial patterns in airway composition as a result of alterations in the maturation, differentiation, and function of airway epithelial cells.

Mechanical shear stress

Under normal conditions, airway epithelial cells are exposed to shear forces generated by airflow during respiration. Shear stress can influence the development of airway epithelial cells, including their proliferation, differentiation, and function. According to Sidhaye et al. (2008), mechanical stimuli positively influence the regulation of airway physiology. Firstly, shear stress regulates the expression of aquaporin AQP5, a protein crucial for water transport across cell membranes. Furthermore, the application of shear stress to airway epithelial tissue activates the transient receptor potential vanilloid 4 (TRPV4) channel and the voltage-dependent L-type calcium channel (VGCC). Activation of these channels increases the intracellular Ca2+ concentration proportional to the applied stress (Nakahari, 2007 & Sidhaye et al., 2008). Consequently, the increased Ca2+ concentration affects the frequency of the ciliary beat, the coordination of ciliary movement, and thereby enhancing the activity of ciliated cells (Lansley & Sanderson, 1999).

Davidovich et al. (2011) observed that wall shear stress, generated by airflow over the airway epithelium, contributes to increased mucus production in the proximal airways. Subsequent studies demonstrated that shear stress influences the basal secretion of mucin by goblet cells (Zhu et al., 2015). The results suggest that an increase in airflow causes an immediate and temporary increase in mucin secretion. Furthermore, this suggests that the increased mucus production after birth is also caused by the increase in shear stress. Shear stress in the airway epithelium is higher postnatally compared to prenatally, due to the increased flow velocity and the distinct physical properties of air relative to the amniotic fluid (Ghadiali & Gaver, 2008 & Trieu et al., 2014). These data indicate that shear stress may play an important role in regulating airway physiology and influencing spatial patterns in the composition of airway epithelial cells.

In conclusion, mechanical stimuli related to respiratory movements seem to play a significant role in the regulation of airway physiology. Multiple studies suggested that compressive force, stretch, and shear stress not only influence the maturation and differentiation of epithelial cells but also modulate their function, contributing to airway remodeling. Specifically, the application of mechanical stimuli has been shown to affect the expression of ciliated cell and goblet cell markers through the activation of specific molecular signaling pathways.

Additional factors

In addition to temperature and mechanical forces, other factors forming a gradient along the airway tree include pH, humidity, and the respiratory microbiota. Clary-Meinesz et al. (1998) demonstrated that ciliary beat frequency varies with pH changes, increasing in alkaline environments and decreasing in acidic environments. Thus, an increase in pH results in an increased ciliary beat frequency. Permanent alterations in ciliated cells are observed when pH levels drop below 3 or rise above 11. An abnormal pH can alter the viscosity and properties of mucus produced by goblet cells, impairing their ability to trap and remove foreign particles. Specifically, an acidic environment reduces the negative charge of mucins, causing mucin molecules to aggregate and thereby increasing mucus viscosity (Leal et al., 2017).

Humidity also plays a crucial role in the development of the airway epithelium, affecting the properties of goblet and ciliated cells. Studies indicate that changes in relative humidity influence both mucus viscosity and the ciliary beat frequency of the airway epithelium (Guarnieri et al., 2023). However, the factors and signals responsible for these changes have not yet been identified.

According to Kayongo et al. (2023), changes in the microbiome can induce the expression of mucus genes, thereby causing epithelial remodeling. Conversely, Dickson et al. (2016) demonstrated that remodeling of the airway epithelium, such as increased mucus production, influences the microbiome, leading to an increase in bacterial colonization and consequently an imbalance in the microbiome, known as dysbiosis. This suggests a bidirectional relationship between airway remodeling and microbiome alterations. However, further research is needed to thoroughly investigate this relationship.

In conclusion, factors such as pH, humidity, and microbiome also influence the function of epithelial cells, contributing to airway remodeling. However, further in-depth investigations are necessary to fully understand these effects.

Conclusion

This review provides an overview of the factors that may regulate and influence spatial patterns in the cellular composition of the airway epithelium under homeostatic conditions. To do so, a literature search was conducted on how spatial patterns occur and evolve during the development of the airway epithelium. This search was followed by the identification of factors that establish a similar gradient along the airway tree and the analysis of how these factors may influence spatial patterns.

Two limitations of the study concern the methods used to review and compare the literature. The first limitation concerns the selection process of the articles for analysis. Instead of a systematic approach, the search relied on keyword searches and studies cited in field-specific reviews. This method may not ensure the same level of thoroughness as a systematic

approach, increasing the likelihood of overlooking some studies. The second limitation involves the selection criteria for the articles. The review primarily included studies focusing on temperature and mechanical forces. Other factors, such as pH, humidity, and the microbiome, were mentioned but not thoroughly investigated in this study. Consequently, future research should aim to conduct a more comprehensive analysis of these latter factors.

Despite these limitations, the investigation into factors that establish a gradient has identified several key elements, including temperature, mechanical forces, pH, and humidity. The results demonstrated that variations in these factors alter the spatial patterns of the airways, impacting the functioning of airway epithelial cells. Several studies indicated that the functioning of ciliated and goblet cells varies in response to temperature changes. However, most of the reviewed studies focus on nasal and tracheal airway cells, overlooking the influence of temperature on the proximal section of the airway epithelium. Further research is needed to provide a more comprehensive understanding. Additionally, studies on mechanical forces showed variations in the differentiation, development, and function of airway epithelial cells based on the type of force applied. Notably, stretching forces seem to inhibit goblet cell expression, while compression forces promote mucus production. This suggests a dynamic balance between compression and stretching that influences mucus production and goblet cell density in the airways. Further research is necessary to confirm the relationship between these mechanical forces. Additionally, changes in pH, humidity, and microbiome can influence the cellular composition of the airway epithelium; however, there are not enough studies for an in-depth analysis of these factors.

In conclusion, this study aims to highlight the importance of understanding how gradient-forming factors influence spatial patterns of cellular composition, with the potential to identify solutions for airway remodeling, a hallmark of many chronic lung diseases. This analysis reveals knowledge gaps in the literature, highlighting the need to better understand the underlying mechanisms of regulation. In line with the discussed results and revealed gaps, I will carry out my MSc thesis focusing on how temperature variations affect the composition and cellular organization of the airway epithelium. Next, the effect of temperature on the mucociliary function of the epithelium will be analyzed, with a focus on ciliary beat frequency, clearance surface, and directionality of ciliary movement. Furthermore, the effect of temperature on the host's defense response to molecular patterns associated with pathogens, including viruses and bacteria, will be examined. Overall, the MSc thesis will aim to provide a detailed overview of the effects of temperature on the airway epithelium to identify potential therapeutic targets for airway remodeling.

Search strategy

The primary studies for this literature review were identified through a search on PubMed, one of the largest biomedical digital databases. Subsequently, Scopus and Google Scholar were also used. The keywords employed included 'airway epithelial tree', 'spatial distribution', 'remodeling', 'temperature', 'mechanical forces', 'pH', 'humidity', and 'microbiome'. These terms were selected based on a preliminary literature review and recommendations from experts in the field. Initially, articles were screened based on their titles and abstracts, followed by a full-text review. The inclusion criteria comprised English-language studies utilizing computational models or in vitro and in vivo experiments on human or mouse cells.

References

Aros, C. J., Pantoja, C. J., & Gomperts, B. N. (2021). Wnt signaling in lung development, regeneration, and disease progression. *Communications Biology*, *4*(1), 601. https://doi.org/10.1038/s42003-021-02118-w

Clary-Meinesz, C., Cosson, J., Huitorel, P., & Blaive, B. (1992). Temperature effect on the ciliary beat frequency of human nasal and tracheal ciliated cells. *Biology of the Cell*, *76*(3), 335–338. https://doi.org/10.1016/0248-4900(92)90436-5

Clary-Meinesz, C., Mouroux, J., Cosson, J., Huitorel, P., & Blaive, B. (1998). Influence of external pH on ciliary beat frequency in human bronchi and bronchioles. *European Respiratory Journal*, *11*(2), 330–333. https://doi.org/10.1183/09031936.98.11020330

Collin, A. M., Lecocq, M., Detry, B., Carlier, F. M., Bouzin, C., De Sany, P., Hoton, D., Verleden, S., Froidure, A., Pilette, C., & Gohy, S. (2021). Loss of ciliated cells and altered airway epithelial integrity in cystic fibrosis. *Journal of Cystic Fibrosis*, *20*(6), e129–e139. https://doi.org/10.1016/j.jcf.2021.09.019

D'Amato, M., Molino, A., Calabrese, G., Cecchi, L., Annesi-Maesano, I., & D'Amato, G. (2018). The impact of cold on the respiratory tract and its consequences to respiratory health. *Clinical and Translational Allergy*, *8*, 1-8. https://doi.org/10.1186/s13601-018-0208-9

Danopoulos, S., Alonso, I., Thornton, M. E., Grubbs, B. H., Bellusci, S., Warburton, D., & Alam, D. A. (2018). Human lung branching morphogenesis is orchestrated by the spatiotemporal distribution of ACTA2, SOX2, and SOX9. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *314*(1), L144–L149. https://doi.org/10.1152/ajplung.00379.2017

Danopoulos, S., Shiosaki, J., & Alam, D. A. (2019). FGF signaling in lung Development and Disease: human versus mouse. *Frontiers in genetics*, *10,* 170. https://doi.org/10.3389/fgene.2019.00170

Davidovich, N. E., Kloog, Y., Wolf, M., & Elad, D. (2011). Mechanophysical stimulations of mucin secretion in cultures of nasal epithelial cells. *Biophysical Journal*, *100*(12), 2855–2864. https://doi.org/10.1016/j.bpj.2011.04.040

Davis, J. D., & Wypych, T. P. (2021). Cellular and functional heterogeneity of the airway epithelium. *Mucosal Immunology*, *14*(5), 978–990. https://doi.org/10.1038/s41385-020-00370-7

Dean, C. H., & Snelgrove, R. J. (2018). New Rules for Club Development: New Insights into Human Small Airway Epithelial Club Cell Ontogeny and Function. *American Journal of Respiratory and Critical Care Medicine*, *198*(11), 1355–1356. https://doi.org/10.1164/rccm.201805-0925ed

Deng, L., Ma, P., Wu, Y., Ma, Y., Yang, X., Li, Y., & Deng, Q. (2020). High and low temperatures aggravate airway inflammation of asthma: Evidence in a mouse model. *Environmental pollution (Barking, Essex : 1987)*, *256*, 113433. https://doi.org/10.1016/j.envpol.2019.113433

Dickson, R. P., Erb-Downward, J. R., Martinez, F. J., & Huffnagle, G. B. (2016). The Microbiome and the Respiratory Tract. *Annual review of physiology*, *78*, 481–504. https://doi.org/10.1146/annurev-physiol-021115-105238

Eenjes, E., Tibboel, D., Wijnen, R. M., & Rottier, R. J. (2022). Lung epithelium development and airway regeneration. *Frontiers in Cell and Developmental Biology*, *10, 1022457*. https://doi.org/10.3389/fcell.2022.1022457

Fagan, K. A., McMurtry, I. F., & Rodman, D. M. (2001). Role of endothelin-1 in lung disease. *Respiratory Research*, *2*, 1-12. https://doi.org/10.1186/rr44

Fang, Y., Liu, H., Huang, H., Li, H., Saqi, A., Qiang, L., & Que, J. (2020). Distinct stem/progenitor cells proliferate to regenerate the trachea, intrapulmonary airways and alveoli in COVID-19 patients. *Cell Research*, *30*(8), 705–707. https://doi.org/10.1038/s41422-020-0367-9

Francis, R. J. B., Chatterjee, B., Loges, N. T., Zentgraf, H., Omran, H., & Lo, C. W. (2009). Initiation and maturation of cilia-generated flow in newborn and postnatal mouse airway. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *296*(6), L1067–L1075. https://doi.org/10.1152/ajplung.00001.2009

Gaillard, D. A., Lallement, A. V., Petit, A. F., & Puchelle, E. S. (1989). In vivo ciliogenesis in human fetal tracheal epithelium. *American Journal of Anatomy*, *185*(4), 415–428. https://doi.org/10.1002/aja.1001850405

Ganesan, S., & Sajjan, U. S. (2013). Repair and remodeling of airway epithelium after injury in chronic obstructive pulmonary disease. *Current Respiratory Care Reports*, *2*, 145–154. https://doi.org/10.1007/s13665-013-0052-2

Giesbrecht, G.G. (1995). The respiratory system in a cold environment. *Aviation, space, and environmental medicine,* 66(9), 890-902.

Ghadiali, S. N., & Gaver, D. P. (2008). Biomechanics of liquid–epithelium interactions in pulmonary airways. *Respiratory Physiology & Neurobiology*, *163*(1–3), 232–243. https://doi.org/10.1016/j.resp.2008.04.008

Gong, F., Yang, Y., Wen, L., Wang, C., Li, J., & Dai, J. (2021). An Overview of the Role of Mechanical Stretching in the Progression of Lung Cancer. *Frontiers in cell and developmental biology*, *9*, 781828. https://doi.org/10.3389/fcell.2021.781828

Guarnieri, G., Olivieri, B., Senna, G., & Vianello, A. (2023). Relative humidity and its impact on the immune system and infections. *International Journal of Molecular Sciences*, *24*(11), 9456. https://doi.org/10.3390/ijms24119456

Han, A., Deng, S., Yu, J., Zhang, Y., Jalaludin, B., & Huang, C. (2023). Asthma triggered by extreme temperatures: From epidemiological evidence to biological plausibility. *Environmental research*, *216*(Pt 2), 114489. https://doi.org/10.1016/j.envres.2022.114489

Henriksson, J. T., Coursey, T. G., Corry, D. B., De Paiva, C. S., & Pflugfelder, S. C. (2015). IL-13 stimulates proliferation and expression of mucin and immunomodulatory genes in cultured conjunctival goblet cells. *Investigative Ophthalmology & Visual Science*, *56*(8), 4186-4197. https://doi.org/10.1167/iovs.14-15496

Hiemstra, P. S., McCray, P. B., Jr, & Bals, R. (2015). The innate immune function of airway epithelial cells in inflammatory lung disease. *The European respiratory journal*, *45*(4), 1150–1162. https://doi.org/10.1183/09031936.00141514

Jeffery, P. K. (1998). The development of large and small airways. *American Journal of Respiratory and Critical Care Medicine*, *157*(5), S174–S180. https://doi.org/10.1164/ajrccm.157.5.rsaa-1

Jeffery, P. K. (2001). Remodeling in asthma and chronic obstructive lung disease. *American Journal of Respiratory and Critical Care Medicine*, *164*, S28–S38. https://doi.org/10.1164/ajrccm.164.supplement_2.2106061

Jorissen, M., & Bessems, A. (1995). Influence of culture duration and ciliogenesis on the relationship between ciliary beat frequency and temperature in nasal epithelial cells. *European Archives of Oto-rhino-laryngology*, *252*, 451–454. https://doi.org/10.1007/bf02114748

Kayongo, A., Robertson, N. M., Siddharthan, T., Ntayi, M. L., Ndawula, J. C., Sande, O. J., Bagaya, B. S., Kirenga, B., Mayanja-Kizza, H., Joloba, M. L., & Forslund, S. K. (2023). Airway microbiome-immune crosstalk in chronic obstructive pulmonary disease. *Frontiers in immunology*, *13*, 1085551. https://doi.org/10.3389/fimmu.2022.1085551

Khosravi, M., Lin, R., Maskey, A. P., Pandey, S., Lin, A., & Lee, L. (2021). A distinct difference between air and mucosal temperatures in human respiratory tract. *Frontiers in Medicine*, *8, 650637*. https://doi.org/10.3389/fmed.2021.650637

Kitterman, J. A. (1996). The effects of mechanical forces on fetal lung growth. *Clinics in Perinatology*, *23*(4), 727–740. https://doi.org/10.1016/s0095-5108(18)30205-7

Lansley, A. B., & Sanderson, M. J. (1999). Regulation of airway ciliary activity by ca 2+ : simultaneous measurement of beat frequency and intracellular Ca 2+. *Biophysical Journal*, *77*(1), 629–638. https://doi.org/10.1016/s0006-3495(99)76919-5

LaPrad, A. S., Lutchen, K. R., & Suki, B. (2013). A mechanical design principle for tissue structure and function in the airway tree. *PLOS Computational Biology*, *9*(5), e1003083. https://doi.org/10.1371/journal.pcbi.1003083

Leal, J., Smyth, H. D., & Ghosh, D. (2017). Physicochemical properties of mucus and their impact on transmucosal drug delivery. *International Journal of Pharmaceutics*, *532*(1), 555–572. https://doi.org/10.1016/j.ijpharm.2017.09.018

Lee, S. G., Lee, S., Baek, J., Yoon, J., & Lee, H. (2021). Mechanical compression enhances ciliary beating through cytoskeleton remodeling in human nasal epithelial cells. *Acta Biomaterialia*, *128*, 346–356. https://doi.org/10.1016/j.actbio.2021.04.030

Li, M., Li, Q., Yang, G., Kolosov, V. P., Perelman, J. M., & Zhou, X. D. (2011). Cold temperature induces mucin hypersecretion from normal human bronchial epithelial cells in vitro through a transient receptor potential melastatin 8 (TRPM8)–mediated mechanism. *Journal of Allergy and Clinical Immunology*, *128*(3), 626-634. https://doi.org/10.1016/j.jaci.2011.04.032

Li, M., Yang, G., Zhou, X., Tselluyko, S., & Perelman, J. M. (2013). The pathophysiological mechanisms underlying mucus hypersecretion induced by cold temperatures in cigarette smoke-exposed rats. *International Journal of Molecular Medicine*, *33*(1), 83–90. https://doi.org/10.3892/ijmm.2013.1535

Lin, L., Zeng, H., Luo, Y., Chen, D., Ma, X., Chen, H., Song, X., Wu, H., & Li, S. (2023). Mechanical stretch promotes apoptosis and impedes ciliogenesis of primary human airway basal stem cells. *Respiratory Research*, *24*(1), 237. https://doi.org/10.1186/s12931-023-02528-w

Man, W. H., De Steenhuijsen Piters, W. A., & Bogaert, D. (2017). The microbiota of the respiratory tract: gatekeeper to respiratory health. *Nature Reviews. Microbiology*, *15*(5), 259–270. https://doi.org/10.1038/nrmicro.2017.14

Mecklenburg, C., Mercke, U., Håkansson, C., & Toremalm, N. (1974). Morphological changes in ciliary cells due to Heat exposure a scanning electron microscopic study. *Cell and Tissue Research*, *148*(1), 45-56. https://doi.org/10.1007/bf00224317

Nakahari, T. (2007). Regulation of ciliary beat frequency in airways: shear stress, ATP action, and its modulation. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *292*(3), L612–L613. https://doi.org/10.1152/ajplung.00433.2006

Nardo, L., Maritz, G., Harding, R., & Hooper, S. B. (2000). Changes in lung structure and cellular division induced by tracheal obstruction in fetal sheep. *Experimental Lung Research*, *26*(2), 105–119. https://doi.org/10.1080/019021400269907

Nawroth, J. C., Roth, D., van Schadewijk, A., Ravi, A., Maulana, T. I., Senger, C. N., van Riet, S., Ninaber, D. K., De Waal, A. M., Kraft, D., Hiemstra, P. S., Ryan, A. L., & van Der Does, A. M. (2023). Breathing on chip: Dynamic flow and stretch accelerate mucociliary maturation of airway epithelium in vitro. *Materials Today Bio*, *21*, 100713. https://doi.org/10.1016/j.mtbio.2023.100713

Nikolaizik, W., Hahn, J., Bauck, M., & Weber, S. (2020). Comparison of ciliary beat frequencies at different temperatures in young adults. *ERJ Open Research*, *6*(4). https://doi.org/10.1183/23120541.00477-2020

Nino, G., Gutierrez, M. J., & Rodriguez-Martinez, C. E. (2022). Human neonatal and infant airway epithelial biology: the new frontier for developmental immunology. *Expert Review of Respiratory Medicine*, *16*(2), 145–147. https://doi.org/10.1080/17476348.2022.2027757

Park, J., & Tschumperlin, D. J. (2009). Chronic intermittent mechanical stress increases MUC5AC protein expression. *American Journal of Respiratory Cell and Molecular Biology*, *41*(4), 459–466. https://doi.org/10.1165/rcmb.2008-0195oc

Polikepahad, S., Haque, M., Francis, J., Moore, R. M., & Venugopal, C. S. (2008). Characterization of endothelin receptors in the peripheral lung tissues of horses unaffected and affected with recurrent airway obstruction. *Canadian Journal of Veterinary Research, 72*(4), 340.

Raby, K. L., Michaeloudes, C., Tonkin, J., Chung, K. F., & Bhavsar, P. K. (2023). Mechanisms of airway epithelial injury and abnormal repair in asthma and COPD. *Frontiers in Immunology*, *14,* 1201658. https://doi.org/10.3389/fimmu.2023.1201658

Rajavelu, P., Chen, G., Xu, Y., Kitzmiller, J. A., Korfhagen, T. R., & Whitsett, J. A. (2015). Airway epithelial SPDEF integrates goblet cell differentiation and pulmonary Th2 inflammation. *The Journal of Clinical Investigation*, *125*(5), 2021–2031. https://doi.org/10.1172/jci79422

Rokicki, W., Rokicki, M., Wojtacha, J., & Dżeljijli, A. (2016). The role and importance of club cells (Clara cells) in the pathogenesis of some respiratory diseases. *Polish Journal of Cardio-thoracic Surgery, 13*(1), 26–30. https://doi.org/10.5114/kitp.2016.58961

Roth, D., Şahin, A. T., Ling, F., Senger, C., Quiroz, E., Calvert, B., Van Der Does, A., Güney, T. G., Tepho, N., Glasl, S., Van Schadewijk, A., Von Schledorn, L., Olmer, R., Kanso, E., Nawroth, J. C., & Ryan, A. L. (2023). Structure-function relationships of mucociliary clearance in human airways. *bioRxiv (Cold Spring Harbor Laboratory)*. https://doi.org/10.1101/2023.12.24.572054

Shaykhiev, R., & Crystal, R. G. (2014). Early events in the pathogenesis of chronic obstructive pulmonary disease. smoking-induced reprogramming of airway epithelial basal progenitor cells. *Annals of the American Thoracic Society*, *11 Suppl 5*(Suppl 5), S252–S258. https://doi.org/10.1513/annalsats.201402-049aw

Sidhaye, V. K., Schweitzer, K. S., Caterina, M. J., Shimoda, L., & King, L. S. (2008). Shear stress regulates aquaporin-5 and airway epithelial barrier function. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(9), 3345–3350. https://doi.org/10.1073/pnas.0712287105

Smith, C. M., Hirst, R. A., Bankart, M. J., Jones, D. W., Easton, A. J., Andrew, P. W., & O'Callaghan, C. (2011). Cooling of cilia allows functional analysis of the beat pattern for diagnostic testing. *Chest*, *140*(1), 186–190. https://doi.org/10.1378/chest.10-1920

Song, K. S., Yoon, J., Kim, K. S., & Ahn, D. W. (2012). c-Ets1 inhibits the interaction of NF-κB and CREB, and downregulates IL-1β-induced MUC5AC overproduction during airway inflammation. *Mucosal Immunology*, *5*(2), 207–215. https://doi.org/10.1038/mi.2011.67

Toskala, E., Smiley-Jewell, S. M., Wong, V. J., King, D., & Plopper, C. G. (2005). Temporal and spatial distribution of ciliogenesis in the tracheobronchial airways of mice. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *289*(3), L454–L459. https://doi.org/10.1152/ajplung.00036.2005

Trieu, D., Waddell, T. K., & McGuigan, A. P. (2014). A microfluidic device to apply shear stresses to polarizing ciliated airway epithelium using air flow. *Biomicrofluidics*, *8*(6), 064104. https://doi.org/10.1063/1.4901930

Tsao, P., Vasconcelos, M., Izvolsky, K. I., Qian, J., Lu, J., & Cardoso, W. V. (2009). Notch signaling controls the balance of ciliated and secretory cell fates in developing airways. *Development*, *136*(13), 2297–2307. https://doi.org/10.1242/dev.034884

Tsao, P., Wei, S., Wu, M., Huang, M., Lin, H., Lee, M., Lin, K. M., Wang, I., Kaartinen, V., Yang, L., & Cardoso, W. V. (2011). Notch signaling prevents mucous metaplasia in mouse conducting airways during postnatal development. *Development*, *138*(16), 3533-3543. https://doi.org/10.1242/dev.063727

Tschumperlin, D. J., Shively, J. D., Kikuchi, T., & Drazen, J. M. (2003). Mechanical Stress Triggers Selective Release of Fibrotic Mediators from Bronchial Epithelium. *American Journal of Respiratory Cell and Molecular Biology*, *28*(2), 142–149. https://doi.org/10.1165/rcmb.2002-0121oc

Varner, V. D., Gleghorn, J. P., Miller, E., Radisky, D. C., & Nelson, C. M. (2015). Mechanically patterning the embryonic airway epithelium. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(30), 9230–9235. https://doi.org/10.1073/pnas.1504102112

Varricchi, G., Brightling, C. E., Grainge, C., Lambrecht, B. N., & Chanez, P. (2024). Airway remodelling in asthma and the epithelium: on the edge of a new era. *The European Respiratory Journal*, *63*(4), 2301619. https://doi.org/10.1183/13993003.01619-2023

Veerati, P. C., Mitchel, J. A., Reid, A. T., Knight, D. A., Bartlett, N. W., Park, J., & Grainge, C. L. (2020). Airway mechanical compression: its role in asthma pathogenesis and progression. *European Respiratory Review: an official journal of the European Respiratory Society*, *29*(157), 190123. https://doi.org/10.1183/16000617.0123-2019

West, J. B. (1978). Regional differences in the lung. *Chest*, *74*(4), 426-437. https://doi.org/10.1378/chest.74.4.426

Wirtz, H. R., & Dobbs, L. G. (2000). The effects of mechanical forces on lung functions. *Respiration physiology*, *119*(1), 1–17. https://doi.org/10.1016/s0034-5687(99)00092-4

Whitsett, J. A. (2018). Airway epithelial differentiation and mucociliary clearance. *Annals of the American Thoracic Society*, *15*(Suppl 3), S143–S148. https://doi.org/10.1513/annalsats.201802-128aw

Xia, G., Tawhai, M. H., Hoffman, E. A., & Lin, C. L. (2010). Airway wall stiffening increases peak wall shear stress: a fluid-structure interaction study in rigid and compliant airways. *Annals of biomedical engineering*, *38*(5), 1836–1853. https://doi.org/10.1007/s10439-010-9956-y

Yaghi, A., & Dolovich, M. B. (2016). Airway epithelial cell cilia and obstructive lung disease. *Cells*, *5*(4), 40. https://doi.org/10.3390/cells5040040

Yang, J., Zuo, W., Fukui, T., Chao, I., Gomi, K., Lee, B., Staudt, M. R., Kaner, R. J., Strulovici-Barel, Y., Salit, J., Crystal, R. G., & Shaykhiev, R. (2017). Smoking-Dependent Distal-to-Proximal repatterning of the adult human small airway epithelium. *American Journal of Respiratory and Critical Care Medicine*, *196*(3), 340–352. https://doi.org/10.1164/rccm.201608-1672oc

Zajac, M., Dreano, E., Edwards, A., Planelles, G., & Sermet-Gaudelus, I. (2021). Airway Surface Liquid pH Regulation in Airway Epithelium Current Understandings and Gaps in Knowledge. *International Journal of Molecular Sciences*, *22*(7), 3384. https://doi.org/10.3390/ijms22073384

Zemke, A. C., Snyder, J. C., Brockway, B. L., Drake, J. A., Reynolds, S. D., Kaminski, N., & Stripp, B. R. (2009). Molecular staging of epithelial maturation using secretory Cell–Specific genes as markers. *American Journal of Respiratory Cell and Molecular Biology*, *40*(3), 340–348. https://doi.org/10.1165/rcmb.2007-0380oc

Zhu, Y., Abdullah, L. H., Doyle, S. P., Nguyen, K., Ribeiro, C. M. P., Vasquez, P. A., Forest, M. G., Lethem, M. I., Dickey, B. F., & Davis, C. W. (2015). Baseline Goblet Cell Mucin Secretion in the Airways Exceeds Stimulated Secretion over Extended Time Periods, and Is Sensitive to Shear Stress and Intracellular Mucin Stores. *PloS One*, *10*(5), e0127267. https://doi.org/10.1371/journal.pone.0127267