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# Graphical abstract

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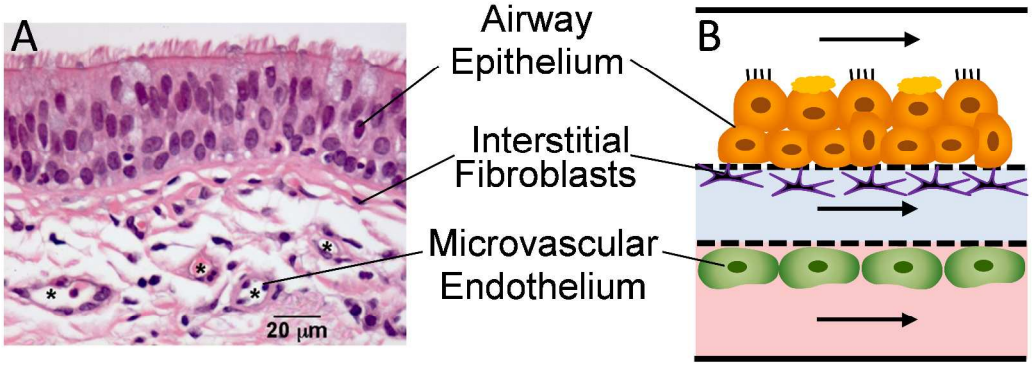
A microfluidic model with three vertically stacked compartments separated by membranes was developed to mimic the human airway mucosa.



# A biomimetic multicellular model of the airways using primary human cells

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**Abstract**

Microfluidic cell cultures enable investigation of complex physiological tissue properties and functionalities. For convenience, they are often implemented with immortalized cell lines, but primary cells more closely approximate the *in vivo* biology. Our aim was to develop a biomimetic microfluidic model of the human airway using all primary cells. The model is comprised of airway epithelial cells cultured at an air-liquid interface, lung fibroblasts and polarized microvascular endothelial cells, respectively positioned in three vertically stacked, individually accessible compartments separated by nanoporous membranes. We report device fabrication, a gravity fed microfluidic system, and culture medium able to support functional co-cultures of all three primary human cell types. As characterized by imaging and permeability measurements, airway epithelial cells in microfluidic devices displayed mucociliary differentiation and barrier function. Subjacent fibroblasts and microvascular endothelial cells were added under conditions enabling co-culture for at least 5 days. Microfluidic airway models based on primary human cells in a relevant biomimetic configuration will improve physiological relevance and will enable novel disease modeling and drug development studies.

## Introduction

The human conducting airways are a primary site of exposure to environmental agents, and are involved in major disorders including rhinosinusitis, asthma, and chronic obstructive lung disease. Our understanding of complex cellular events occurring in response to inhaled agents and during development of airway diseases is limited, in part, by the current status of airway models. To enhance studies of disease pathogenesis and for drug discovery, a growing body of research aims to capture the complexity of tissues and organs using microfluidic multi cell type models [1]. Biomimetic microfluidic lung culture models have been engineered to reproduce physiological properties such as phasic mechanical expansion and edema formation [2-4]. However, these models rely on immortalized cell lines, which are convenient, but do not reproduce critical *in vivo* phenotypes such as mucus secretory and multi ciliated cells in the airway or type 1 and 2 pneumocytes in the alveoli [5]. Primary airway epithelial (AE) cells, when cultured on porous supports at an air liquid interface (ALI) become pseudostratified and differentiate into cell types representative of their normal *in vivo* morphology and physiology [6]. These cultures reproduce *in vivo* properties such as high resistance to gene therapy vectors [7] and cell-type specific virus infection [8] and are considered more relevant for studies of drug absorption [9], inhalation toxicology [5], epithelial repair and remodeling and disease pathogenesis [10, 11]. Three dimensional, well differentiated *in vitro* organoid cultures of primary bronchial and alveolar epithelial cells in Matrigel® have been reported [12, 13] but these do not allow for convenient microfluidic access to the different tissue compartments.

Integration of the epithelium with other airway cells is central to both normal physiology and pathologic responses. The airway mucosa consists of an epithelial surface with underlying interstitial cells and capillaries that act as a functional trophic unit [14], which is involved in disease pathogenesis. For example, in asthma, airway remodeling includes subepithelial fibrosis, angiogenesis, and endothelial dysfunction associated with edema and enhanced inflammatory cell influx (reviewed in [15]). The thickness of the interstitial compartment decreases as bronchi become bronchioles, becoming reduced to a fraction of a micron in the gas exchanging alveoli. Augmenting primary lung epithelial cell ALI cultures with fibroblasts (Fb) and lung microvascular endothelial (MvE) cells, representing the interstitial and capillary compartments, respectively, would be a step towards greater physiologic complexity and relevance.

Co-cultures of lung epithelial and endothelial cells on opposite sides of a nanoporous membrane have been reported, but were limited to non-differentiated primary alveolar type II cells [16] or a bronchial epithelial cell line in submerged culture [17]. Co-cultures including differentiated primary AE cells and fibroblasts have been reported, either on a membrane or with fibroblasts in a subjacent extracellular matrix [18, 19]. The few reported examples of triple co-cultures include a mixture of two cell types in one of the two compartments divided by a membrane [20, 21].

Our goal was to develop a biomimetic multicellular construct more closely recapitulating the airway mucosal microarchitecture, including interfaces between three primary cell types. We created a dual membrane, integrated microfluidic device emulating the *in vivo* tissue structure and enabling heterotypic cell interactions, while maintaining cell compartmentalization (Fig. 1). This configuration enables barrier-forming AE and MvE cells to be grown on nanoporous membranes, whose material, coating and pore size can be separately optimized. The distance separating AE and MvE cells is defined by design, and recapitulates the human airway interstitial thickness. Independent fluidic access enables compartment-specific cell type seeding and probing. We report: (1) the design and fabrication of the multicompartment device; (2) a novel membrane bonding process; (3) a passive long-term fluidic perfusion system; (4) conditions for primary, well-differentiated AE cell ALI culture; (5) characterization of AE cell barrier properties by measuring apparent permeability values; and (6) investigation of medias supporting functional multiple cell type co-cultures. The latter is a major challenge since each primary cell type prefers its own specific media.

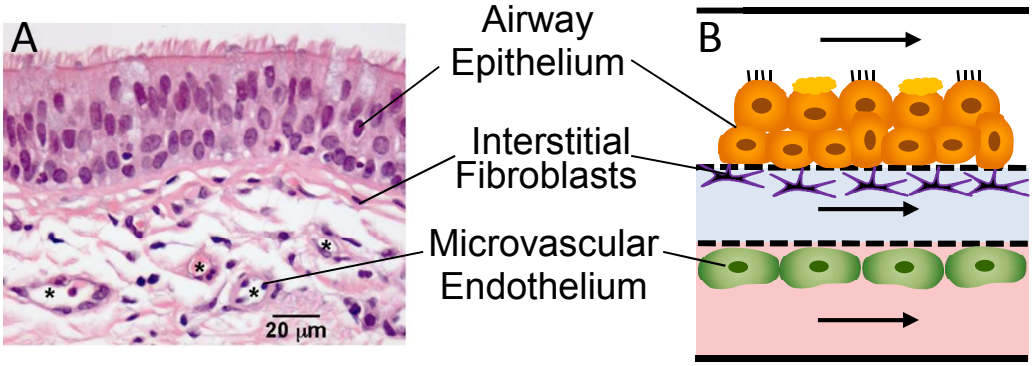


**Fig. 1** (A) Histology cross section of normal human bronchus from a lung transplant donor (hematoxylin and eosin stain). Asterisks mark capillaries. (B) Schematic of the airway mucosa model including three vertically stacked compartments with three different cell types separated by two nanoporous membranes, arrows indicate channels for fluid or air.

## Methods

### Device fabrication

Cells were cultured on a nanoporous membrane support sandwiched between three aligned 10 mm x 1 mm long microfluidic polydimethylsiloxane (PDMS) channels (Fig. 2). The apical and basolateral channel width was 1 mm, the middle layer channel width was 1.2 mm. The height of the apical compartment was 280 μm (ensuring minimal fluid shear stress, $1\times10^{-3}$ dyn/cm$^2$ for a flow rate of 0.1 μl/min). The central and basolateral compartments were typically 150 μm high, although tests were conducted with 280 μm tall compartments as well. Two-layer

devices, including one membrane, were routinely used for experiments involving a single cell type, and a layout using wide compartments (10 mm x 2 mm) was also used.

Silicon masters for molding of PDMS were fabricated by deep reactive ion etching as described elsewhere [22]. Briefly, silicon wafers were patterned and etch depths of 150 µm to 280 µm were obtained by selecting etch times. Following photoresist removal and after dicing, the silicon molds were coated with a fluorosilane anti-adhesion coating by vapor deposition. Sylgard 184 (Dow Corning, from Ellsworth Adhesives) was mixed in a 10:1 ratio, poured onto the silicon masters, and cured at 65°C. The micromolded PDMS was carefully peeled from the silicon masters and inlet/outlet ports were pre-punched with a stainless steel round punch (0.024" x 0.016" x 1.5", Syneo). Fluidic connections were achieved using right angle 22 gauge stainless steel connectors, (SC22/15RA, Instech Laboratories) press fit and glued to the punched holes in the PDMS, and polyurethane tubing (VAHBPU-T25, Instech Laboratories) was connected.

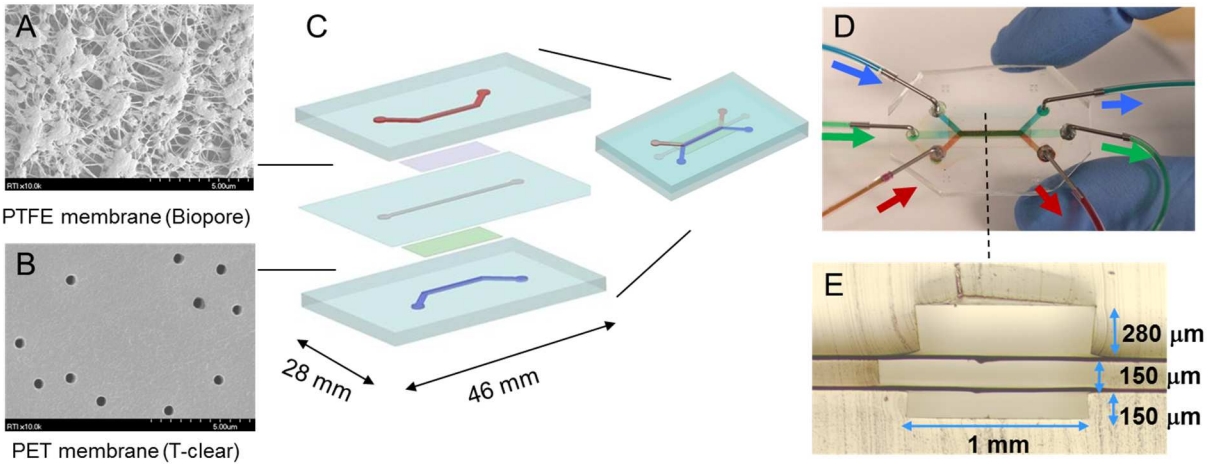


**Fig. 2** Microfluidic device configuration. (A) and (B) SEM images of the PTFE and PET membranes; (C) exploded view and schematic (D) photograph of a 10 x 1 mm device with dyes in the three fluidic channels; (E) optical microscope image of a 10 x 1 mm device cross section.

## Membrane bonding

Membrane-integrated microfluidic devices were fabricated using three different types of membranes: hydrophylized polytetrafluoroethylene (PTFE) cut from a 10 foot x 1 foot Biopore® sheet (BGCM00010, 0.4 µm pore size, Millipore); polyester (PET) cut from 0.4 µm pore size T-clear Transwells® 3450, Corning; and polycarbonate (PC), 1 µm pore (Cyclopore Thin Clear, 7091-4710, Whatman). PET and PC membranes were bonded using wet aminosilanization of the membrane and contact bonding with plasma-treated PDMS [22, 23]. The membranes were treated with oxygen plasma (200W, MVD, Applied Microstructures) for 5 minutes, then immediately soaked in a 5% aqueous solution of aminopropyltrimethoxysilane (APTMS, SIA6011, Gelest) that had been preheated to 80°C, for 20 minutes and rinsed in deionized water

prior to contact bonding to PDMS. The hydrophylized PTFE membranes required the development of a custom procedure for bonding to PDMS. Oxygen plasma treatment and/or silanization of the hydrophylized PTFE membranes were found to degrade the membrane resulting in loss of wet-ability and inability to bond to PDMS. We achieved bonding by adopting aminosilanization of PDMS, which has been reported to enable bonding to plasma treated polymer membranes [24]. After rinsing by sonication for 20 minutes in DI water, the silanized PDMS was placed on a Teflon film and dried at 80 °C for approximately 15 minutes, or until no moisture remained on the surface and used within 24 hours. The hydrophylized PTFE was left untreated (i.e., not exposed to plasma) and sandwiched between two silanized PDMS layers and aligned under a stereoscope. Small water droplets from a 22 gauge syringe were placed at the four corners of the PDMS layer to re-wet it prior to bonding. Thermocompression by a 200 g weight at 65°C overnight (on a Teflon film wrapped device) was required to achieve bonding of PTFE membrane devices (Fig. 3). In order to ensure leakage free operation of PTFE membrane devices seeded with cells in an incubator, subsequent high temperature heat treatment at 121 °C for 30 minutes with no compression in an autoclave was necessary.

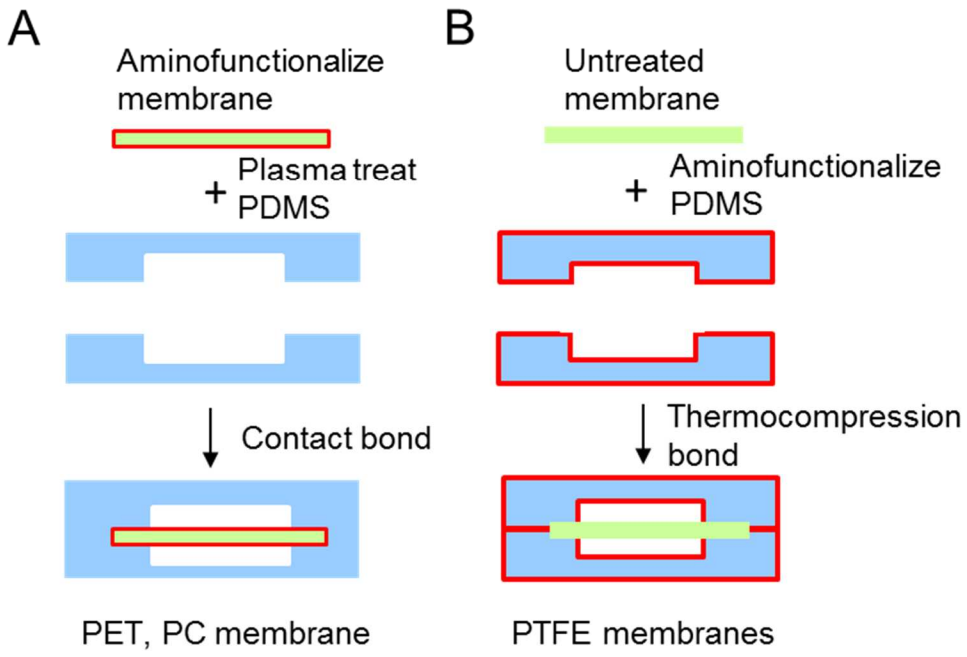


**Fig. 3** Membrane bonding process (A) for polyester (PET) and polycarbonate (PC) membranes and (B) for PTFE membrane.

The fabrication of devices with three vertically stacked channels and two membranes used the same membrane bonding procedure developed for single membrane devices. The micromolded PDMS thin film for the middle layer (Fig. 2C) was obtained by lamination on a kapton film carrier by a custom apparatus described elsewhere [22]. In the case of heterogeneous

membrane devices (e.g. with a PTFE and PET membrane), the bonding of the two membranes was carried out sequentially. The PET membrane was bound first between the bottom PDMS and the thin middle PDMS on a kapton carrier. The thin PDMS films formed a robust bond to PET membranes when used within 5 minutes from plasma treatment. The laminate was then thermocompressed at 65°C for a time of at least 10 hours with a weight of at least 500g. After thermocompression, the Kapton film was released by soaking the laminate device in an isopropanol bath. The second membrane was then bonded between a top PDMS layer and the already joined middle and bottom layers, with a second thermocompression at 65°C for a time of at least 10 hours with a weight of 500g.

**Cell Culture**

Human lungs unsuitable for transplantation were obtained under protocol #03-1396 approved by The University of North Carolina at Chapel Hill Biomedical Institutional Review Board. Tracheo-bronchial epithelial cells (AE) were obtained and cultured as previously described [25]. Human lung microvascular endothelial cells were obtained by dispase and elastase digestion of peripheral lung tissue stripped of the visceral pleura, followed by primary culture in EGM-2 MV media plus FBS (Lonza, Allendale, NJ). The cells were subjected to 2-3 rounds of CD31 bead purification (Dynabeads, Life Technologies/Invitrogen, Carlsbad, CA), after which they were >95% CD31-positive as measured by flow cytometry and immunostaining.

Primary human lung fibroblasts were obtained by outgrowth from finely minced distal human lung tissues onto scratched type I/III collagen coated dishes in DMEMH media plus 10% fetal bovine serum, penicillin and streptomycin plus supplementary antibiotics and antimycotics. Primary isolated cells were obtained by treatment with trypsin/EDTA and were subcultured as above but without supplementary antibiotics and antimycotics. The passaged cells exhibited typical fibroblast morphology and were negative for CD31 and pan-cytokeratin as assessed by flow cytometry. As described below, cells were cultured both on nanoporous membranes in microfluidic devices and in commercially available well inserts.

Primary human AE cells were expanded in BEGM media and used for air-liquid interface cultures at passage 1 or 2. AE were cultured apically on either 12 mm Millicell® inserts with 0.4 μm pore hydrophylized PTFE membranes (Millipore, PICM-01250) or 6.5 mm Transwell® inserts with 0.4 μm pore PET membranes (Corning Incorporated, 3470), both coated with Collagen type IV (Sigma Aldrich, C7521). AE seeding number was 250,000 for Millicell® and 50, 000 for Transwell®. Cells were grown using either ALI medium 1 (formulation described in [26] or ALI medium 2 [27].

Primary MvE cells were maintained in EGM-2MV medium (Lonza CC-3202) and used between passages 4 and 10. The optimal collagen coating for growing MvE cells on inserts was

found to be different for PET and PTFE membranes. Millipore PTFE inserts were coated with collagen I (BD Biosciences, 354249) at 12 μg/cm$^2$ and air-dried prior to UV crosslinking. Transwell PET inserts were coated with collagen type I/III (Advanced BioMatrix PureCol, 5005-B) diluted 1:75 in sterile water and incubated for 2-3 hours at 37ºC. Excess collagen was aspirated and membranes allowed to dry before UV crosslinking. Fibroblasts were maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin and were used between passages 4 and 12. Fibroblasts were grown on Millipore PTFE inserts or Transwell PET inserts coated with Collagen IV or Collagen I. For microfluidic culture, devices were sterilized by autoclaving. For AE cells, the upper surface of the topmost membrane was coated with collagen IV at 0.8 mg/mL and left to dry (for 72 hours) in a sterile hood and then exposed to UV light for 30 minutes, on each side. For Fb and MvE cells, membranes were coated with 1.2 mg/mL collagen I or 0.62 mg/mL collagen I/III, respectively. Prior to seeding, devices were connected to a peristaltic pump (MasterFlex C/L, Cole Parmer) and flushed, at 5 μl/min, with sterile water for 10 minutes, followed by PBS (20 minutes), and finally by media perfusion. Cells were seeded at $4.0\times10^4$cells/μL for AE, $1.3\times10^4$ cells/ μL for Fb, and $1.2\times10^5$ cells/μL for MvE. Cell seeding was carried out by manual injection using a 25 μl Hamilton syringe (60371-011, VWR) fitted with a 22 gauge blunt end needle. An amount of media equal to the volume of inlet tubing and fluidic connector (~15 μl) was first drawn with the syringe, followed by cell suspension corresponding to the volume of the channel to be seeded (~ 2μl or 4 μl). The solution was then gently injected through the inlet tubing.

After cell seeding, devices were positioned in Petri dishes containing a sterile wet paper towel and parafilm sealed to minimize liquid evaporation, and placed in an incubator to allow for cell adhesion. Following cell adhesion, for times ranging from 3.5 hours (Fb cells) to overnight (AE and MvE cells), cell culture devices were connected to a gravity driven media flow system. Once AE cells reached confluence, the apical channel was filled with air to establish air-liquid interface and the lower channels remained under fluid flow to refresh the cell culture medium. For triple co-cultures, the membranes were collagen coated with different collagens simultaneously by filling the top, middle and lower compartment with collagen IV, collagen I and collagen I/III respectively and letting them dry as described above.

**Operation of the microfluidic devices**

A custom system for gravity-driven flow was developed using commercial 10 ml "constant flow syringes" (DN/5 Constant Flow Syringe, Harvard apparatus) set in custom supports holding 2 or 4 syringes (Thorlabs bases and posts supporting machined polycarbonate plates) (Fig. S1). These syringes consist of two nested static syringes dripping into each other to provide a constant medium height difference (ΔH) between inlet and outlet. Stability of the flow was established by securing a polyester wick (TX1009 Texwipe) in the outlet syringe, at a fixed height, to drain the outlet reservoir to a third waste container located at a lower level (Fig. S1A). Ethylene oxide sterilized syringes were connected to cell culture devices via tubing (VAHBPU-

T25, Instech Laboratories ) connected a stainless steel constrictions of 150 μm inner diameter (U-1222, Idex). The use of the wick overcame tubing resistances present in the system and ensured that flow was uninterrupted. The wick and the reservoir were wrapped in parafilm to minimize evaporation. Bubble formation causing flow restriction was observed on average every few days and addressed by manual removal.

**Cell culture characterization**

Fluorescence and phase contrast images were acquired with an inverted microscope (Olympus IX71), using either a 4X or long working distance 20X objective, by a CCD camera (Hamamatsu C4742-80-12Ag) and commercial imaging software (ImagePro). Confocal images of immunostained samples were obtained on a Olympus FV1000 laser scanning confocal in the Michael Hooker microcopy facility at the University of North Carolina at Chapel Hill. Live/Dead staining was performed with a kit according to the manufacturer's instructions (L-3224, Life Technologies), using a 1:1000 dilution for both the calcein and ethidium dyes. Hoechst (H3570, Life Technologies) was used as a nuclear stain, also at a 1:1000 dilution in phosphate buffered saline PBS. Image analysis to quantify viability was carried out in ImageJ and live and dead cell numbers were normalized to the total cell number as determined by the nuclear stain. Immunostaining was performed *in situ* for both cell inserts and microfluidic devices. AEs were rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature. Samples were then rinsed with TBS, permeabilized in 0.2% Triton X-100 in 1X TBS for 30 minutes, rinsed in TBS, and then blocked for one hour with a solution of 1% BSA, 1% fish gelatin, 0.1% Triton X-100, and 5% normal goat serum (S-1000, Vector Laboratories, Burlingame, CA) in TBS, at room temperature. Samples were then stained with rat monoclonal anti-tubulin (MAB 1864, Millipore), mouse monoclonal anti-Mucin 5AC/Gastric Mucin AB-1 (ThermoScientific, 45M1), and a rabbit polyclonal anti-MUC5B (a kind gift from Dr. John Sheehan, University of North Carolina at Chapel Hill) overnight at 4 °C. Primary antibodies were used at up to 5 μg/mL for monoclonal, and 3 μg/mL for polyclonal antibodies. After primary antibody incubation, samples were rinsed with staining diluent solution (25% blocking buffer in 1X TBS) and then incubated with secondary antibodies (1:1000 dilution in diluent) for 2 hours at room temperature, away from light. Secondary antibodies were purchased from Jackson Immuno Research. After secondary antibody incubation, samples were washed with 1X TBS. Samples were counterstained with Hoechst (1:1000 in PBS), and then washed in PBS prior to imaging.

F-Actin staining was used for all three cell types. Cells were washed with cold PBS then fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature. After fixation cells were washed with PBS. Next, samples were blocked with 0.075% Triton X-100 and 1% BSA in TBS for 30 minutes at room temperature. Blocking buffer was removed and an Alexafluor-conjugated phalloidin antibody (Molecular Probes) was added at a 1:40 dilution in blocking buffer for 20 minutes at room temperature. Finally, cells were washed with PBS and incubated with Hoechst as described above.

Histological sections were obtained for AE cells cultured on inserts. AE cells from nine different donors were cultured in parallel on PET membranes (Snapwell) or hydrophilized PTFE membrane (Millicell) at an air liquid interface. Histological sections of paraffin embedded samples followed by hematoxyin/eosin staining were made as described previously [28] and cultures of the same age on the different substrates were compared. Cell layer thickness from at least three regions of each section was measured using transmission optical microscopy.

The barrier integrity of the AE cultures was characterized by measuring the transport of 20 kDa FITC-dextran (Sigma), used at concentration of 3 mg/ml. On cell inserts, transepithelial electrical resistance (TEER) measurements were also conducted using a commercial system (EVOM 2, World Precision Instruments) and chopstick electrodes (STX-2). TEER values were determined by subtracting the baseline resistance measured in the absence of cells and multiplying by the cell culture area.

For AE cultures in microfluidic devices the apical compartment was filled with dextran solution and a blank HBSS buffer solution was flowed in the basolateral compartment at 0.5 μl/min. Effluent from the basolateral compartment was collected at 30 minute intervals for 150 minutes. In static culture, 400 μL of dextran solution was added to the apical surface and 600 μL of blank buffer added to the basolateral surface. Samples of 100 μL were collected at 30 minute intervals for 180 minutes. The level of fluorescence in the collected media was measured using a fluorometric image plate reader (Flipr Tetra, Molecular Devices) and converted to solute mass (in μg) according to solute standards measured at the same time. The amount of transported solute (ΔQ) was obtained by summing the transported solute mass over time. The apparent permeability coefficient was calculated using the equation $P_{app}= (\Delta Q/\Delta T)/(A*C_0)$, where $\Delta Q/\Delta T$ is the slope of the linear portion of the compound transported vs. time curve, A is the area of the device, and $C_0$ the concentration.

## RESULTS

### Culture of individual cell types in microfluidic devices.

Seeding and culture conditions for AE, MvE, and Fb cells were optimized individually using two-layer, single membrane devices and cultures in inserts for comparison. The membrane collagen coating for microfluidic device was selected based on optimal cell growth and surface marker expression on culture inserts. After seeding and adhesion in microfluidic devices with a 10 mm x 1 mm cell area, the cells were connected to the gravity driven flow system (Supplementary information, Fig. S1) with a target flow rate of 0.1 μL/min. The cells were kept under continuous flow for nutrient replenishment for the duration of the culture, up to 35 days. The passive fluidic perfusion approach enabled autonomous operation for over 3 days between media refills.

Well-differentiated AE cultures were obtained on both 0.4 μm pore size, collagen IV coated PET or hydrophylized PTFE membranes inserts, but we noted that the cells were sensitive to the membrane type. Hydrophylized PTFE in Millicell inserts was preferable because the cells achieved mucociliary differentiation more quickly (typically 21 days on PTFE compared to 28 days on PET in ALI 1 medium) and consistently exhibited a thicker cell layer than on PET, more closely emulating the *in vivo* morphology (Fig. S2). Average cell layer thickness on histological cross sections was 19±4 μm for PTFE and 9.9 ±1.7 μm for PET (n=9) when comparing cultures from the same donor. We therefore developed a fabrication process for PTFE membranes in microfluidic devices. The same hydrophylized PTFE membrane material used in Millicell inserts is available as a roll from the manufacturer, and it can be conveniently cut to shape. Combining silanization and thermocompression enabled fabrication of irreversibly bonded microfluidic devices that supported leak-free cell culture up to 5 weeks. PTFE integrated devices were characterized for bond strength by air burst test, including measurements after 21 days of operation with media in an incubator. The PTFE device burst pressure was lower than that of PDMS devices without membranes or polycarbonate membrane integrated devices (Table S1), but was adequate for leak-free performance, including manual rinses and peristalstic pump sample collections.

Passage 1 or 2 primary AE cells from 5 different donors were seeded on collagen IV-coated PTFE membranes in a 280 μm tall microfluidic compartment in ALI 1 medium, which is known to produce mucociliary cell differentiation with tight barrier properties in static cultures [26]. In the PTFE-membrane integrated microfluidic device, AE cells produced a confluent monolayer within a widely ranging period from 2 days to 14. A tight polygonal morphology comparable that of AE on Millicell inserts was achieved (Fig. 4 A,B). Over time, the AE cells grew to a pseudostratified epithelium, which resulted in blurry images because cells are in multiple focal planes (Fig. 4C).

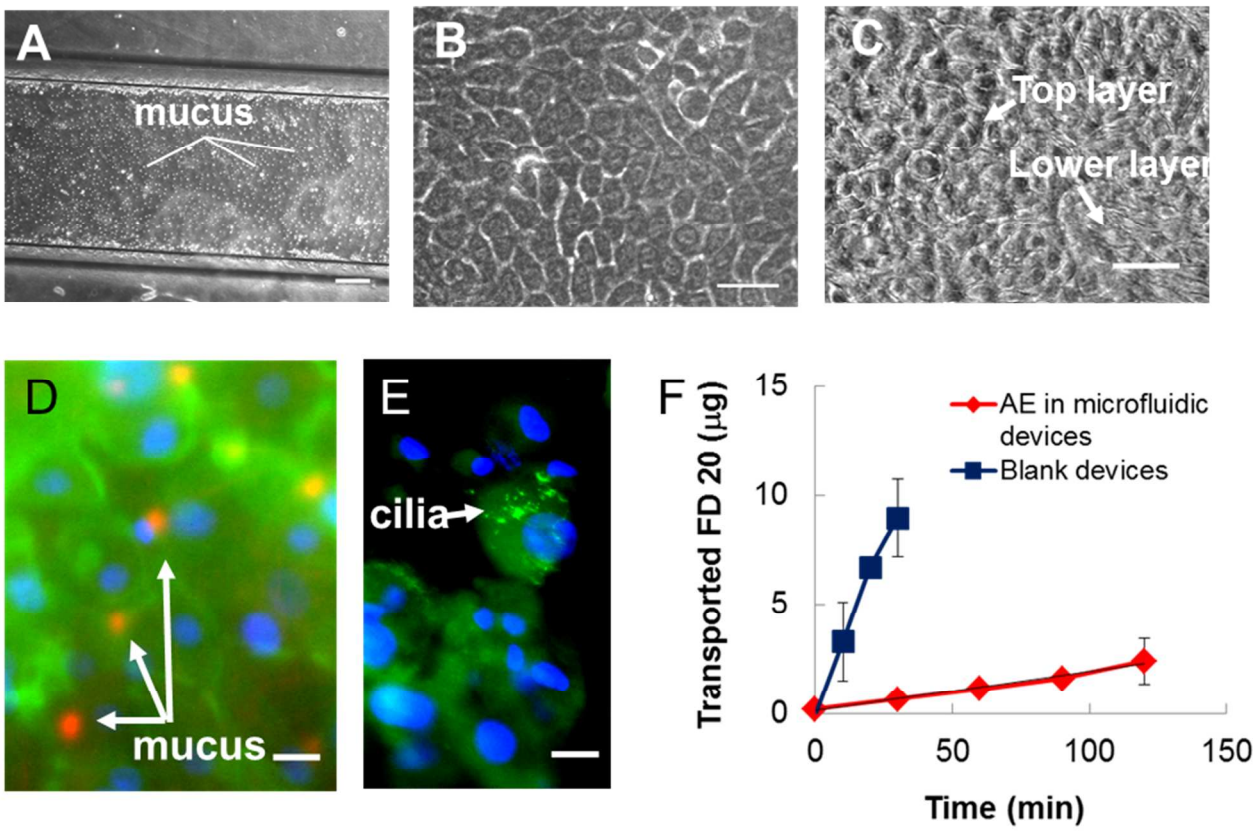


**Fig. 4** AE cells in microfluidic devices. Phase contract images at: (A) 4x magnification image at day 17; arrows point to recently exocytosed granular material assumed to be mucin granules. Scale bar=200 μm. (B) 20x magnification image of a confluent monolayer of cells at day 10 illustrating the typical tight-packed polygonal morphology. (C) 20x magnification image of a pseudostratified culture at day 28, blurriness is caused by cells in different focus planes. Scale bar= 50 μm. Differentiation and functionality of AE in microfluidic devices. Epifluorescence images in paraformaldehyde fixed samples (D) Mucus staining (red), phalloidin (green) and Hoescht (blue); (E) tubulin staining (green) as marker of cilia; scale bar=20 μm. (F) Permeability measurement: apical to basolateral transport of FITC-dextran 20 in cultures $P_{app}=9x10^{-7}$ cm/sec (n=3) and blank devices.

Mucin granule exocytosis is the first marker of differentiation and it was routinely visualized in microfluidic devices within two weeks (Fig. 4A and D). Cilia were detected on selected cultures by either movement or tubulin marker staining (Fig. 4E) at culture days ranging from 15 to 35 days. Permeability studies were performed in cultures at day 28 to 35 in ALI 1 medium. The transport of FITC-dextran 20 is shown in Fig. 4F and corresponds to $P_{app}=9x10^{-7}$ cm/sec (n=3). This permeability is significantly different than the values measured for blank devices indicating the presence of a barrier with cell junction formation and proper sealing by the cellular layers of the edges of the cultures. However, AE in microfluidic devices were more permeable than cultures in inserts which exhibited $P_{app}=2.3\pm0.6 \times 10^{-8}$ cm/sec (n=4) at day 21. In

spite of the selection of optimal membrane substrate, AE cells in microfluidic devices were systematically delayed in reaching the steps of differentiation with respect to their static well counterpart, and exhibited poorer barrier performance.

Fibroblasts in AE and MvE specific media proliferated and maintained an elongated morphology (Fig.S3) on both PET and PTFE membranes coated with either collagen IV or collagen I. Fibroblasts seeded at $2.5\times10^4$ cells/μL in a 150 μm tall compartment of a microfluidic device typically reached confluence within 3 days.

At the same seeding density of 1.6 $10^6$ cell/$cm^2$ MvE cells in EGM medium typically reached confluence at day 2 on PET membranes in Transwells while they required 4 days on Millicell PTFE membranes. Thus, PET membranes were selected for MvE support in microfluidic devices. MvE were seeded on the underside of collagen I-III coated PET membrane devices in a 150 μm tall compartment and left to adhere overnight prior to establishing flow. MvE cells formed a very thin layer and were difficult to distinguish on a membrane support by phase contrast microscopy. F-actin immunostaining illustrated MvE cells in a confluent, tightly packed layer at day 7 in a microfluidic device with a PET membrane (Fig. S3).

**Co-culture medium selection**

Specific serum-free media is required for AE cell differentiation at an air-liquid interface. In order to determine the optimal medium for co-culture we investigated the effect of AE media on Fb and MvE cells. Fibroblasts acquired a spindle shape morphology in all of the medias tested, namely ALI1, ALI2 and EGM media. We qualitatively observed that proliferation was slower than in DMEM/10% FBS medium and slow proliferation is desirable in a co-culture with contact-inhibited and barrier-forming cells.

For MvE cells the following co-culture medium options were investigated and compared to EGM: ALI1, ALI2, 50:50 ALI1:EGM, 50:50 ALI2:EGM. We evaluated cell morphology by phase contrast of live cells, f-actin staining, cell viability, and cell number /area (Table 1 and Fig. S4). MvE culture in ALI2 medium were similar to those in 50:50 ALI2:EGM medium, so the latter was not evaluated further. ALI2 medium includes UltroserG, which is a serum substitute. MvE cell viability was greater than 94% in all media, except ALI 1 (Table 1). However, an increased MvE cell diameter was observed in all media compared to their optimized EGM media and, in non-EGM media, the confluent MvE layer had a significantly lower number of cells per area. Immunostaining also showed that small gaps between cells were more frequent in non-EGM media. These results suggest that further optimization of medias for co-culture of multiple cell types will be useful.

Table 1. Effect of medium on MvE in well plates (average ± standard deviation, n=3 wells).

| Medium | Cell number/area (#/mm$^2$) | Viability (%) |
|---|---|---|
| EGM | 322.± 13 | 98±0.2 |
| ALI 1 | 25 ± 2 | 70± 12 |
| ALI 1:EGM 50:50 | 154 ± 8 | 96 ± 1.8 |
| ALI 2 | 97 ± 17 | 94± 5 |

Based on cell density results (Table 1), two medias, 50:50 ALI1:EGM and 100% ALI2, were further evaluated in AE and MvE cell co-cultures in Millicell inserts. MvE cells were seeded on the underside of three week old, differentiated AE cultures with TEER values > 200 Ohm*cm$^2$. While less physiological than a three layer configuration, TEER can be easily monitored longitudinally in inserts to determine media suitability. We note that TEER values are dominated by the AE versus MvE components of bilayer co-cultures, typically exhibiting resistances >500 Ohm*cm$^2$ and <30 Ohm*cm$^2$, respectively, in their preferred medias. Fig. 5a shows a typical longitudinal TEER study of AE/MvE cell co-cultures in 50:50 ALI1:EGM medium. The drop in TEER values is attributed to AE cells rounding up and ultimately exfoliating.

The dextran permeability of AE cells alone in ALI 1 ($P_{app}$=2.3±0.6 x $10^{-8}$ cm/sec, n=2 wells) and AE/MvE cell co-cultures in 50:50 ALI1:EGM ($P_{app}$=4±1 x$10^{-8}$ cm/sec, n=4 inserts) was comparable when measured after 2 days. However, at day 4 in 50:50 ALI1:EGM, the co-culture TEER decreased and $P_{app}$ was two orders of magnitude more permeable, indicating cell culture degradation and loss of barrier functionality.

TEER remained consistent over several days in media ALI 2 (200-300 Ohm*cm$^2$ are typical with this medium) and, at day 5, dextran permeability was comparable to the AE only cultures (Fig. 5b) indicating that the functional barrier properties are maintained in co-cultures for at least 5 days with this medium. After permeability experiments, day 5 co-cultures were fixed and stained and both mucus and cilia differentiation markers were observed while a MvE cell layer was present on the bottom side of the membrane (Fig. 5C,D).

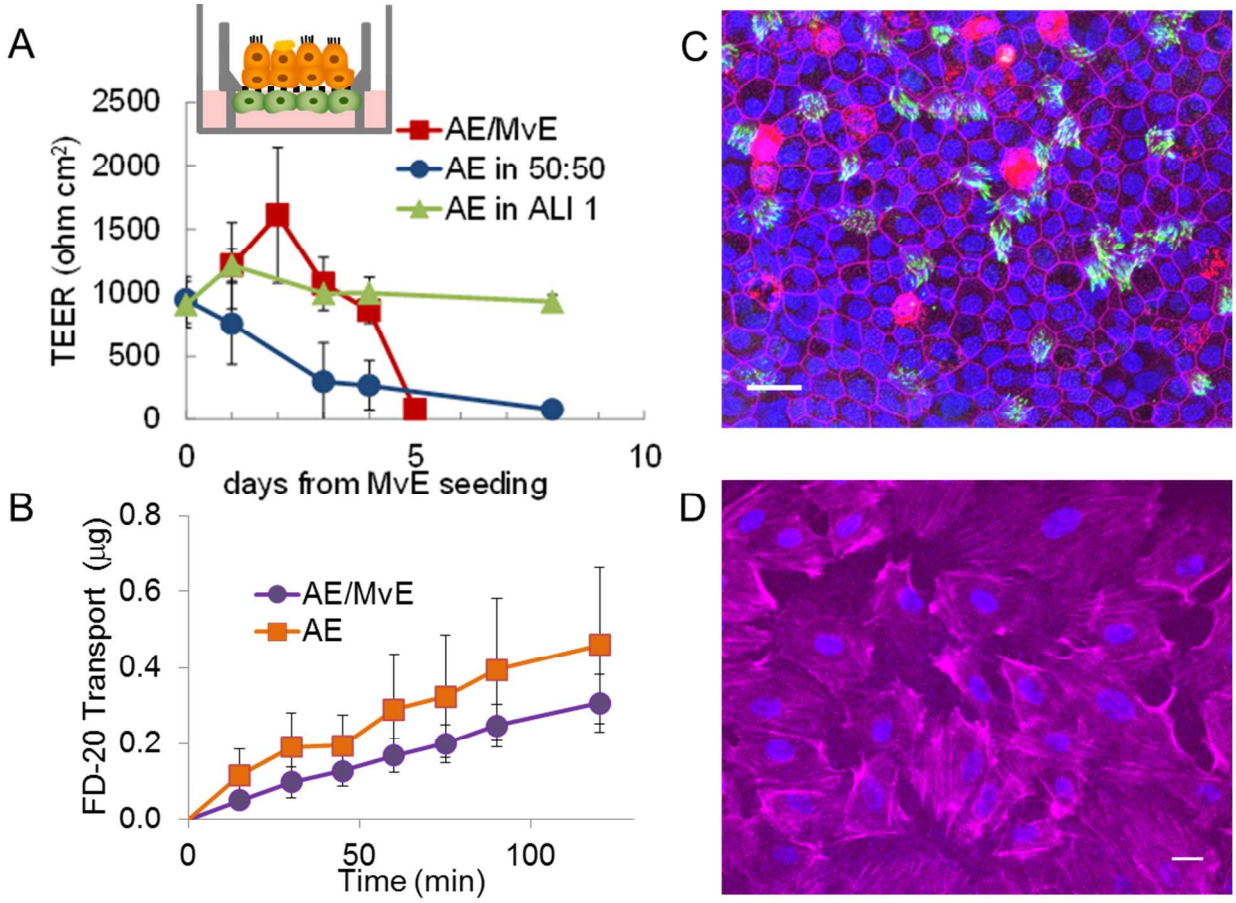


**Fig. 5** AE/MvE co-culture medium optimization. (A) Schematic of experiments performed in Millicell inserts and TEER measurements in ALI 1 and 50:50 ALI1:EGM. (B) Permeability of FITC-dextran MW 20 kDa, at day 5 in ALI 2; $P_{app}$=6.7±1.8 x$10^{-8}$ cm/sec, (n=3) for AE/MvE and $P_{app}$=10±3.8 x$10^{-8}$ cm/sec for AE cell (n=3). (C) and (D) Confocal microscope image of AE/MvE on an excised insert membrane in ALI 2. (C) AE and (D) MvE culture from the same optical field imaged at a different z stack position. Mucin (red), Cilia (green), Phalloidin (magenta) and nuclei (blue). Scale bar: 50µm.

### Co-culture in microfluidic devices

Three-compartment 10 mm x 1 mm microfluidic devices were fabricated with a PTFE membrane for the AE/Fb cells and a PET membrane for MvE cells. Collagen IV was used for AE and Fb on the PTFE membrane, and collagen I-III was used on the PET membrane in the basolateral compartment for MvE.

The AE cells were seeded first in the device and were typically grown for 10 days in ALI 1 medium. The device was flipped upside down and Fb cells were seeded next in the middle

compartment in ALI 1 media. After overnight culture, MvE were seeded and ALI 1 medium in the device was replaced by either ALI 2 or 50:50 ALI1:EGM medium. Optimal co-culture results with all three cell types in confluent layers were obtained in ALI 2 medium. Immunostaining, imaged by epifluorescence and confocal microscopy, demonstrated individual cell layers for each of the three cell types present at different focal planes (Fig. 6). An X-Z vertical confocal view showed separation between the cells grown on the two membranes and highlighted the thickness of the upper AE and Fb cell layer (Fig. 6B). An epifluorescence microscope view of the same device taken with a long working distance 20x objective at different focal planes enabled independent visualization of the three cell types. Phalloidin staining outlined the different cell morphologies. The differentiated, multilayer AE culture exhibited mucin granules, fibroblasts were elongated and spindle-shaped, and cobblestone-like MvE cells were present on the underside of the lower membrane (Fig. 6C, D, and E).

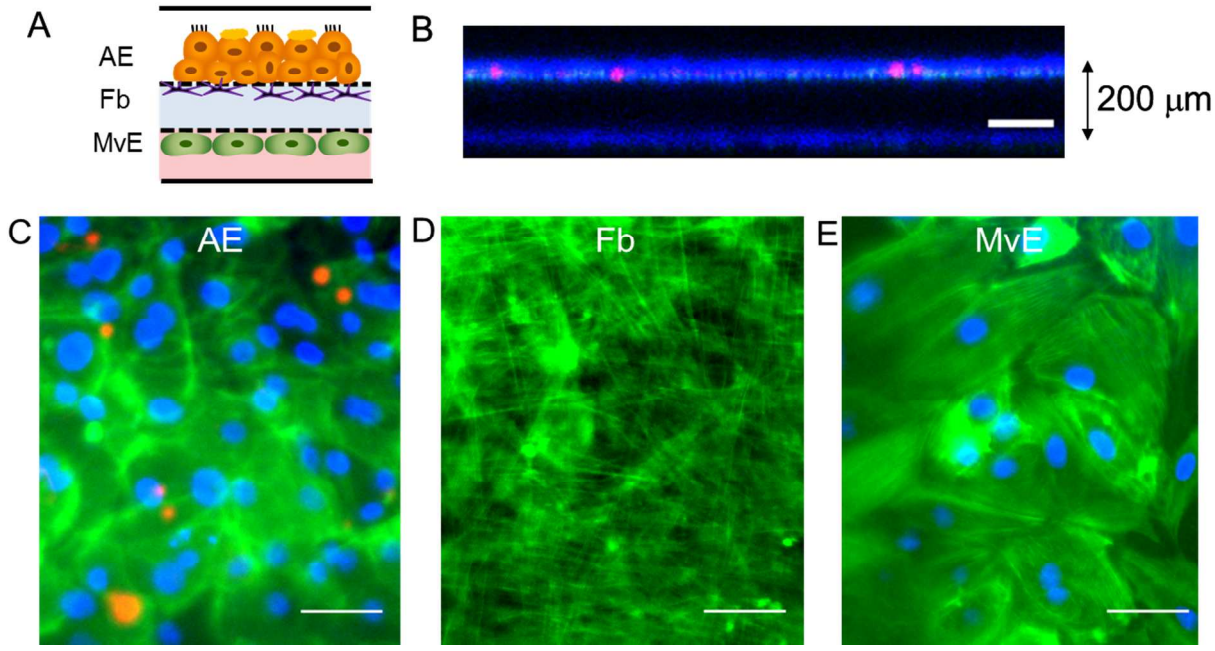


**Fig. 6** Triple co-culture of differentiated primary airway cells in ALI 2 medium. (A) Schematic of the structure. (B) vertical cross section of fluorescently stained device imaged by confocal microscope at low magnification. (C) AE, (D) Fb and(E) MvE cell layers epifluorescence images acquired with a long working distance 20x objective from the same optical field. (Hoechst nuclear stain, green phalloidin and red mucin, Hoechst not shown in D for image clarity). Scale bar 50 μm.

## Discussion

Microfluidic devices enable cell culture in an accessible, controlled spatial arrangement. We leveraged this property to develop a multi-compartmental construct with

three vertically stacked culture chambers emulating the microarchitecture of the airway mucosa. The device features physiologically relevant geometrical separation between cell types and enables heterotypic cell interaction by paracrine signaling across nanoporous membranes. While co-cultures with two cell types are possible in commercially available permeable culture supports, the triple co-culture recapitulating relationships between the airway mucosa epithelium, interstitium and vasculature require an engineered perfused middle layer.

Several challenges were overcome to achieve a tripartate co-culture using all primary cells, including well-differentiated AE cells. Given the multiple week time period required for AE cell differentiation, we employed a hydrophilized PTFE upper membrane that supports more rapid development of a thick, well-differentiated epithelium (Fig. S2). We report a novel method to bond this membrane in PDMS microfluidic devices, which will likely be beneficial for other microfluidic cell culture systems. We note that this membrane material is available in bulk, preventing the common practice of cutting membranes from costly prefabricated commercial cell culture supports. We demonstrated that primary AE cells adhered to this membrane *in situ* and persisted in long term cultures (> 3 weeks) that achieved mucociliary differentiation as reported for their static culture counterparts. A previous microfluidic air-liquid interface culture of primary nasal epithelial cells was obtained in a modified static Transwell culture grown for 5 weeks prior to integration of the apical surface with a microfluidic channel for gas exposure [29]. Growing AE cells in microfluidic devices from initial seeding is more flexible, enabling integration in constructs with varying dimensions. We noted that cell differentiation for all donor specimens was slower in 10 x 1 mm microfluidic devices than on comparable 12 mm diameter commercial culture supports. We recently observed faster confluence and development of a stable air-liquid interface in 10 x 2 mm microfluidic format. The precise mechanism is unknown, but systematic investigation of culture geometry is a promising avenue for further optimizing the microfluidic culture. Different membrane coatings could also be explored, since this work selected the coating for each cell type based on optimal results from cultures in insert which may not correspond to the optimal microfluidic culture condition

Because long-term culture is needed for AE cell differentiation, we developed a passive fluidic perfusion approach for cell nutrient replenishment that enables autonomous operation for over 60 hours as well as operation of parallel devices. While suboptimal because of the occasional flow stoppage due to bubble formation, the gravity driven flow system described here enables carrying the devices from the incubator to the microscope for daily observation without breaking the flow connection as necessary when using bulky syringe pumps and without adding contamination risks.

Finding a common culture medium for multiple primary cells is challenging. We found two solutions for bilayer AE and MvE cell cultures. Simply mixing the two individually preferred medias (ALI1 and EGM) 50:50 only offers a short time window for

experiments in co-culture because construct functionality begins to degrade after day 2. An alternative media, ALI2, which contains the proprietary serum substitute UltroserG that requires a USDA import permit, supports AE and MvE co-culture for at least five days. Using appropriate timing of Fb and MvE cell seeding, we demonstrated triple co-culture feasibility and imaged mucociliary epithelial differentiation in ALI2 media. However, in this compromise media, the MvE cell monolayer did not have the same compact cobblestone morphology observed in the preferred EGM media. Further optimization of media for long-term co-culture of multiple primary cell types clearly required. In this regard, we noted that 2.5% FBS in EGM was necessary for MvE cell adherence and growth to a confluent cobblestone monolayer even in EGM media, and we preliminarily found that ALI1+2.5% FBS supports both AE survival and MvE growth similar to ALI2 media.

The aim of this study was to develop a culture configuration that recapitulates the physiological tissue interfaces of the airway mucosa. A further improvement of the described microfluidic airway model would be the culture of the interstitial compartment fibroblast cells in an extracellular matrix environment. This matrix could be perfused with media supporting both AE epithelial cells at an air-liquid interface and hardy fibroblast cells, and may be achieved using a hydrogel cage to contain mechanically fragile extracellular matrices [22]. This approach would improve physiological fidelity and provide a more realistic cellular milieu. This microfluidic airway mucosa co-culture model could also be extended to different cells types, for example mast cells and smooth muscle cells, to obtain more representative models of common conditions such as allergy or asthma.

## Conclusion

A microfluidic model with three vertically stacked compartments separated by nanoporous membranes was developed to mimic the airway mucosa microarchitecture. The device was designed to support culture of primary AE cells at an air liquid interface, and primary fibroblasts, and MvE cells in the three compartments, respectively. A novel bonding strategy for hydrophylized PTFE membranes was developed because this nanoporous material provided optimal growth support for AE cells. A fluidic perfusion approach for cell nutrient replenishment that required no external power and enabled autonomous operation for over 3 days as well as parallelization of device operation was demonstrated. Well-differentiated primary human tracheo-bronchial epithelial cells at an air-liquid interface that had physiological functionalities including barrier properties were demonstrated in a microfluidic device. Medias enabling AE/MvE co-culture were investigated, and a medium supporting well-differentiated AE cells with barrier property functionality for 5 days was identified. A microfluidic triple co-culture was achieved using an appropriate co-culture medium and cell seeding order. This work demonstrates that a microfluidic device can support culture of primary airway epithelial cells and illustrates a co-culture approach enabling heterotypic cell interaction while maintaining

compartmentalization. Primary cells and the multi-cellular biomimetic configuration offer improved physiological relevance and the described microfluidic model can be used for a wide range of applications to study the biology and pathophysiology of the airway mucosa.

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