# Enhancement of airway epithelial cell differentiation by pulmonary endothelial cell co-culture

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## Abstract

Cross-talk between lung epithelial cells and their microenvironment has an important physiological role in development. Using an *in vitro* model of differentiation of human induced pluripotent stem cells (iPSCs) to air–liquid interface (ALI)-cultured lung epithelial cells, we investigated the contribution of the microenvironment to maintenance of the lung progenitor cell state. Our protocol modeled in vivo cell-to-matrix and cell-to-cell interactions. These included growth of iPSCs on inserts coated with different basement membrane proteins (collagen I, IV, fibronectin, heparan sulfate or Matrigel plus collagen IV) and co-culture with human pulmonary microvascular endothelial cells (HPMECs). Marker gene expression was measured by RT-qPCR and protein expression and localization was confirmed by immunocytochemistry. The results showed that iPSCs grown on collagen IV had the highest success rate in terms of differentiation to robust ALI-cultured lung epithelial cells, followed by fibronectin, collagen I and heparan sulfate. Coating with Matrigel mixed with collagen IV further increased the success rate to > 97 %. Co-culture of iPSCs with HPMECs enhanced the expression of key airway lineage markers (NKX2.1, KRT5, TP63, MUC5AC, MUC16, FOXJ1, CFTR and SCGB1A1) during ALI culture. Cross-talk between iPSCs and their microenvironment during cell differentiation had a significant effect on lung epithelial cell differentiation in these 3D *in vitro* models. Both matrix proteins and endothelial cells play critical roles in the differentiation of lung progenitor cells.

## Keywords

Air–liquid interface; Endothelial cells; Lung progenitor cells; Matrix proteins

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## 1. Introduction

Respiratory diseases such as chronic obstructive pulmonary disease (COPD), asthma and cystic fibrosis (CF) share many common pathophysiologic traits, such as recurrent inflammation, susceptibility to infection and epithelial dysfunction and damage. Therapeutic approaches to all these respiratory diseases may benefit from the use of stem cells, which may be key to increasing our understanding of the molecular and cellular mechanisms underlying disease. In this context, *in vitro* studies on stem cells can make an important contribution to new treatments. Despite the establishment of multiple differentiation protocols to generate lung epithelial cells from human stem cells (Firth et al., 2014; Huang et al., 2014; McCauley et al., 2017; Wong et al., 2015), the generation of functional airway epithelial cells *in vitro* remains challenging.

In the past few years, many protocols were developed to improve the generation of lung epithelial cells from induced pluripotent stem cells (iPSCs) (Jacob et al., 2017; McCauley et al., 2017; Wong et al., 2015; Yamamoto et al., 2017). In general, these are iterative processes involving the stepwise addition of signaling factors to guide iPSCs to become immature, mature and fully functional lung epithelial cells. The first step of the pathways is to differentiate iPSCs into definitive endoderm, for which there is a well-established protocol, and several commercial kits are available. The next developmental steps aim to direct definitive endoderm cells towards a highly enriched anterior-foregut fate population, which involves dual inhibition of the bone morphogenetic protein (BMP) and transforming Growth Factor β (TGF-β) pathways (Green et al., 2011). This approach was adopted by several groups to differentiate pluripotent cells into airway epithelial cells (Dye et al., 2015; Firth et al., 2014; Hawkins et al., 2017; Longmire et al., 2012) others used a combination of fibroblast growth factor 2 (FGF2) and Sonic hedgehog (SHH) signaling (Wong et al., 2012). Recent enhancements of these protocols have focused on the purification of specific differentiated lung epithelial cell types, which have been used successfully to study airway disease (Abo et al., 2020; Hawkins et al., 2021; Wang et al., 2022).

During the early stage of respiratory system development, the primary bronchi begin an orderly program of pseudo-glandular branching morphogenesis. The branched epithelium is then divided into proximal and distal regions. Alveolar type II (ATII) cells are the progenitor cells generated from the latter region that give rise to mature ATII and ATI cells, which are responsible for the functional alveolar unit required for gas exchange (Fehrenbach, 2001). Differentiation of human pluripotent stem cells towards distal lung ATII cells on fibronectin-coated cell culture plates was reported by Huang et al. (2015), and the process was subsequently further improved by using methods of 3D organoid culture (Jacob et al., 2017; Yamamoto et al., 2017). iPSC differentiation towards the proximal fate is more challenging, as it requires more precise spatiotemporal regulation to generate specific lineages of different cell types with distinct functions (see (Calvert and Ryan Firth, 2020) for review). Specific strategies to maximize the efficiency of the differentiation process into lung-specific progenitor cells involve the regulation of pathways of WNT signaling (WNT3a), BMP4 (glycogen synthase kinase 3 inhibitor), and retinoic acid, which are required for accurate lung cell specification (Gotoh et al., 2014; Hawkins et al., 2017; Huang

et al., 2014). The differentiation of iPSCs to airway cells is still imperfect; however, different methods using various combinations of growth factors and inhibitors at defined stages have improved the process.

Optimal iPSC-derived lung cells should retain stem-cell properties and have the capacity to generate multiple disease-relevant cell types, with mature transcriptomes. For example, cystic fibrosis transmembrane conductance regulator (CFTR)-expressing cells are required for investigating functional deficits in cystic fibrosis. It is generally accepted that basal stem cell-like cells (expressing tumor protein *P63* (TP63) and keratin 5 (KRT5)) are the major cell type with self-renewal properties in the airway epithelium. Improvements to methods of differentiating iPSCs to differentiated lung cell types, in addition to basal-like cells, are still required, though recent progress has achieved the robust generation of multi-ciliated and secretory cells (Firth et al., 2014; McCauley et al., 2017; Wong et al., 2015).

Basal cells are dependent on close interactions with the specialized extracellular matrix (ECM) components in basement membranes. ECM proteins include type IV collagen, laminin, entactin, heparan sulfate proteoglycan, and chondroitin sulfate proteoglycan (Sannes and Wang, 1997). Dynamic changes in ECM components have important roles during lung development, including the regulation of airway branching, differentiation of different cell types, and alveolarization. Additional ECM components including other collagens are also involved during lung development (Busch et al., 2021). Endothelial cells (ECs) line the small capillary blood vessels that deliver nutrients, oxygen and cellular components to local tissues including basement membranes. Capillary ECs may also release angiocrine factors into the microenvironment, which may interact reciprocally with other neighboring cell types to regulate lung development (Rafii et al., 2016; Ramasamy et al., 2015).

We previously used functional genomics protocols to identify open chromatin genome-wide and changes in transcriptomic profiles through the differentiation of iPSC to lung epithelial cells at air–liquid interface (ALI) (Kerschner et al., 2020). The mechanisms mediating the interactions between basal cells and the ECM, and crosstalk between airway epithelial cells and ECs, may play important roles in lung development; however, these processes are not well-understood due to the lack of suitable *in vitro* models. Here, we developed an *in vitro* model based upon in our earlier work (Kerschner et al., 2020) to investigate further the impact of i) different matrix protein components in the substrate, and ii) EC-*co*-culture to provide an EC-conditioned microenvironment, on the maintenance of the lung progenitor cell state and differentiation into diverse lung cell types.

## 2. Materials and methods

### 2.1. Cells

Three iPSC lines, ND1.4 (CR0000011; RUCDR Infinite Biologics (https://www.rucdr.org)), CWRU205 (Dr Paul J. Tesar, Dept. of Genetics, Case Western Reserve University), and GM23476 (Coriell Institute), were used for differentiation to lung cells grown at the ALI. Human pulmonary microvascular endothelial cells (HPMECs) from PromoCell (C-12281; Heidelberg, Germany) were cultured in Endothelial Cell Basal Medium MV (PromoCell)

according to the manufacturer's protocol. Human bronchial epithelial (HBE) cells were obtained from the Marsico Lung Institute CF Center Tissue Procurement and Cell Culture Core (University of North Carolina, Chapel Hill, NC) and cultured according to their published protocols (Fulcher et al., 2005; Randell et al., 2011). HBE cells from three donors were grown individually under ALI conditions for 5 weeks and were used as the normalization control for gene expression values generated by RT-qPCR.

### 2.2. iPSC culture

The protocol of iPSC differentiation to lung epithelial cells was initially based on the study of Wong et al. (Wong et al., 2015) with some modifications, and our previous work (Kerschner et al., 2020). First, membranes of Transwell inserts were coated with different matrix proteins (collagen I, collagen IV, fibronectin, heparan sulfate, all at 60 μg/mL, and collagen IV (60 μg/mL) + Matrigel (1/80 dilution) to compare the effects on cell growth. Additionally, 50 nM retinoic acid was added to the culture medium from the start of differentiation to lung progenitor cells at Day 16 of culture. PneumaCult-Ex Medium and PneumaCult-ALI Medium (STEMCELL Technologies, Vancouver, Canada), were used instead of B-ALI differentiation medium and B-ALI growth medium (Lonza) in our original protocol (Kerschner et al., 2020) as the latter was discontinued. The experiments were performed at least twice for each iPSC line (12 inserts per line were used in each). The percentage of NK2 Homeobox 1 (NKX2.1)-positive cells was assessed by flow cytometry at the end of anterior foregut endoderm (AFE) (Wong et al., 2015), and NKX2.1-positive cells were monitored by immunostaining at subsequent stages of differentiation.

### 2.3. RT-qPCR

RT-qPCR was performed by standard protocols. Briefly, total RNA was extracted from cells with TRIzol (Life Technologies), cDNA was synthesized from RNA with random hexamers using a Taqman Reverse Transcription reagents kit (Applied Biosystems) and qPCR experiments were carried out with SYBR Green master mixes. The sequences of the primer pairs specific for each target gene were as used in our previous studies (Kerschner et al., 2020; Mutolo et al., 2018) and are listed in Supplementary Table 1. A pool of RNA from HBE ALI week 5 cultures from three donors (equimolar amounts from each) was used to normalize the expression of lung marker genes. RNA from Caco-2 cells provided the positive control for *HNF4* expression. The expression of the marker genes was normalized to that of beta-2-microglobulin (β*2M*) in each sample, and then compared to the average level of expression of the same gene in HBE ALI cultures from three donors.

### 2.4. Immunohistology

Cells cultured on membrane supports were paraformaldehyde-fixed and paraffin-embedded. After being cut into 5-μm sections, the samples were deparaffinized and rehydrated. Antigen retrieval was performed in sodium citrate buffer (10 mM sodium citrate and 0.05 % Tween 20, pH 6.0) in a 98 °C water bath for 45 min. The sections were then post-fixed in 4 % paraformaldehyde (in PBS) for 15 min, permeabilized with 0.05 % saponin for 10 min, and blocked with 1 % BSA before staining. The sections were subsequently incubated with primary antibody/antibodies at 4 °C overnight. Primary antibodies used were KRT5 (ab52635), NKX2.1 (ab76013) and TP63 (ab735), all from Abcam; and SCGB1A1

antibody (HPA031828) was from Sigma. After three washes with PBS (with 0.05 % Tween 20), the sections were incubated with secondary antibody for 1 h at room temperature. Secondary antibodies were Alexa Fluor 488–conjugated anti-rabbit IgG and Rhodamine Red-X–conjugated anti-mouse IgG (both from Jackson ImmunoResearch). After washing, the samples were nuclear counter-stained with DAPI (4′,6-diamidino-2-phenylindole), mounted with Prolong Antifade Mountant (Invitrogen), and samples were then examined using a Leica DMR microscope.

## 2.5. Statistics

The unpaired *t*-test was used to compare the results between iPSCs with EC co-culture and the control. Statistical analyses were performed with the use of Prism software (Graphpad v.9.3.1). A P-value < 0.05 was considered as statistically significant.

## 3. Results

### 3.1. Extracellular matrix proteins and iPSC differentiation

iPSCs were differentiated to definitive endoderm using a StemDiff definitive endoderm kit (STEMCELL Technologies), then the cells were seeded onto matrix protein-coated membrane supports to differentiate into anterior-foregut endoderm. The cultures were directed towards lung progenitor cells, followed by culture in medium containing FGF7, FGF10 and FGF18 to induce formation of immature lung cells. Maturation and polarization of the cells were carried out under ALI culture.

iPSCs grown according to the original protocol (Wong et al., 2015) with modifications (Kerschner et al., 2020), using a collagen IV coating on the filter inserts did not exhibit a high success rate of complete differentiation after 5 weeks of ALI culture. Success rate was defined as the percentage of healthy cultures produced divided by the total number of membranes for each condition and cell line (one culture = one membrane insert, *n* = 12 for each iPSC line in each experiment). Health cultures polarized on the membrane and generated a multilayered epithelium without medium leakage to the apical side during the ALI culture, and no damage to the culture as observed under phase contrast microscopy. To improve the success rate, the effects of coating the substrate with different ECM components, including collagen I, collagen IV, fibronectin, heparan sulfate, and Matrigel with collagen IV, were examined. Among the three iPSC lines, ND1.4, CWRU205 and GM23476, the success rates of iPSC differentiation to lung cells through ALI culture at weeks 1, 3 and 5 were compared. For the ND1.4 and GM23476 cells *n* = 2 differentiation replicates, and *n* = 3 for CWRU205 cells.

As shown in Fig. 1, at week 1 of ALI culture, cells grew well on most ECM components, with the exception of heparan sulfate. At week 5, membranes with a collagen IV coating had a 78.7 % success rate, followed by 62 % with fibronectin, then collagen I at only 28.7 %; no culture survived on heparan sulfate-coated membranes after 5 weeks of ALI culture. When a mixture of Matrigel (1:80 dilution) and collagen IV (60 μg/mL) was used, the success rate of ALI culture was further improved to 97.2 % (Fig. 1). Among the three iPSC lines, ND1.4 and CWRU205 consistently showed better cell differentiation through ALI culture, forming

a healthier, polarized and multilayered epithelium (data not shown). These two lines were therefore selected for use in subsequent experiments.

### 3.2. Co-culture with pulmonary endothelial cells increased lung marker-positive cells

For the co-culture experiments, HPMECs were introduced into the culture from the stage of expansion of immature lung cells (on day 16). HPMECs were resuspended in 50 % Matrigel/medium at $4 \times 10^5$ cells/mL. Medium from the 12-well inserts with differentiated iPSCs on the upper membrane was removed, leaving a small amount to keep the cells from drying out. The inserts were inverted and placed in a 15-cm Petri dish, and 75 μL of HPMECs in Matrigel were layered onto the reverse side of the membrane. For the controls, 75 μL of 50 % Matrigel without HPMECs were used to coat the reverse side of the inserts. The inserts were kept in a 37 °C incubator for 15 min to allow the Matrigel to set, then placed back in a 12-well plate with 0.5 mL and 1.5 mL of medium added to the upper and lower chambers, respectively. The location of ECs in Matrigel underneath the membrane was confirmed by histological examination of sections through the culture inserts (Supplementary Fig. 1).

Next, the effect of co-culture with HPMECs on the capacity of iPSCs to differentiate into lung cells was investigated by measuring the expression of marker genes. We routinely assessed the percentage of NKX2.1-positive cells by flow cytometry at the end of AFE and observed that >97 % of cells were NKX2.1-positive in all the iPSC lines used (Supplementary Table 2). Expression of the marker genes was firstly normalized to β*2M*, and then compared to the average level of expression in HBE cells cultures at ALI week 5 (average of three donors). When the cells were co-cultured with ECs, a significantly higher level of the lung lineage marker *NKX2.1*, was seen after ALI culture for 1, 3 and 5 weeks (Fig. 2A). The enhanced expression of NKX2.1 was further validated by immunofluorescence using a specific antibody (Fig. 2B and 2C). We routinely assessed NKX2.1-positive cells by immunostaining during the immature lung cells and the ALI stages to ensure the cultures were differentiating appropriately. Furthermore, hematoxylin and eosin staining of the 3D ALI cultures at 1, 3 and 5 weeks showed an abundance of ciliated epithelial cells at all stages (Fig. 2D).

### 3.3. Endothelial cell co-culture increased basal cell marker gene expression

Basal cells are known to be the stem cells that support the differentiation of other cell types, such as secretory/club, goblet or ciliated cells, in the airways. Hence, the effect of EC co-culture on basal cell maintenance and progenitor capacity was assessed next. During the first two weeks of ALI culture, no difference was observed in the expression level of *KRT5*, while EC co-culture significantly increased *KRT5* expression levels after week 3 of ALI culture (Fig. 3A). In contrast, EC co-culture enhanced the *TP63* expression at week 1 to week 3 of ALI culture, after which the stem cell contribution to the culture was about the same as that in the controls (Fig. 3B). Basal cell staining (KRT5 and TP63) was observed in cells adjacent to the basement membrane (Fig. 3C).

### 3.4. Effects of endothelial cell co-culture on gene expression in other (non-basal) cell types

The expression level of several genes encoding proximal airway lineage markers was assayed in ALI cultures differentiated with or without EC co-culture. These markers included mucin 5AC (MUC5AC) for goblet cells, Forkhead box J1 (FOXJ1) for multi-ciliated cells, and mucin 16 (MUC16) and CFTR for surface airway epithelial cells. Ionocytes are rarely observed in these iPSC-derived cultures (Hawkins et al., 2021; Ngan et al., 2021). As shown in Fig. 4, for all these markers, the ALI cultures exhibited comparable or higher expression than the HBE ALI control cultures (which were set to 100 % in normalization calculations); in addition, significantly higher expression, or trends of higher expressions, of *MUC5AC* (Fig. 4A), *MUC16* (Fig. 4B), *FOXJ1* (Fig. 4C) and *CFTR* (Fig. 4D) were observed at week 2 to week 4 of ALI culture for cells co-cultured with pulmonary ECs compared to the controls.

Secretoglobin family 1A member 1 (SCGB1A1) and family 3A member 2 (SCGB3A2) are well characterized markers of club cells in the airway epithelium (Reynolds et al., 2002). *SCGB1A1* and *SCGB3A2* mRNA levels decreased during culture at ALI (Fig. 4E and Supplementary Fig. 2A), and no statistically significant difference was evident upon EC co-culture for the majority of time points (except at ALI week 4 where EC co-culture significantly enhanced *SCGB1A1* expression). Immunostaining for SCGB1A1 confirmed the presence of club cells in the culture (Fig. 4F). There was also no difference in *MUC5B* level between the control and EC-coculture group for all ALI stage (Supplementary Fig. 2B). Hence, though EC co-culture substantially enhances the differentiation from iPSCs of multiple cell types in the ALI cultures it may have less direct impact on club cell differentiation. We also measured the expression level of hepatocyte nuclear factor 4 (*HNF4*), a gastrointestinal/liver marker, in the cultures to confirm that the cell differentiation after the AFE stage was towards airway rather than gut lineages. All the iPSC differentiations through the ALI stages showed very low levels of *HNF4* expression in comparison to the Caco-2 cell positive control (Supplementary Fig. 2C).

## 4. Discussion

In this series of experiments, we optimized the matrix protein substrates to improve iPSC differentiation into lung epithelial cells under ALI culture, and also demonstrated that co-culture of iPSCs with ECs enhanced the expression of key airway lineage marker genes.

Among the individual matrix protein components used, membrane supports coated with collagen IV resulted in the best success rates (~89% at week 3 and ~ 79 % at week 5) for the differentiation of iPSCs to lung epithelial cells under ALI culture (Fig. 1). When a mixture of collagen IV and Matrigel was used, the success rates were further increased (>97 % at both week 3 and week 5). The working concentration of collagen IV for the coating was 60 μg/mL. Matrigel contains enriched ECM components, including laminin, collagen type IV, entactin and perlecan (heparan sulfate proteoglycan). Based on the information provided by the manufacturer, Matrigel contains approximately 30 % collagen IV, which corresponds to about 10 μg/mL of collagen IV in our cultures (as Matrigel was used at a 1/80 dilution). The improved iPSC differentiation to lung epithelial cells resulting from growth with Matrigel

might be due mainly to laminin, which accounts for about 60 % of Matrigel. Heparan sulfate proteoglycan is known to be important for stem cell function (Ravikumar et al., 2020). However, we found that the use of heparan sulfate proteoglycan alone resulted in the poorest cell growth, suggesting that this component alone is not adequate for use in iPSC differentiation to airway cells.

Studies have shown that the intimate crosstalk of ECs with their neighboring cells, such as epithelial cells, mesenchymal cells, and immune cells, is crucial to guide organogenesis in multiple organs, including the lung (Kina et al., 2020), pancreas (Bastidas-Ponce et al., 2017; Pan and Wright, 2011) and liver (Si-Tayeb et al., 2010; Soares-da-Silva et al., 2020). The ECs that compose capillaries also display a heterogeneous morphology and gene expression pattern dependent on their resident tissue. Data from transcriptional profiling of ECs isolated from fetal and adult human organs revealed EC heterogeneity (Marcu et al., 2018), and more recent single-cell RNA sequencing further supported a tissue-specific EC identity (Paik et al., 2020). Lung ECs display significantly upregulated expression of genes associated with immune functions, including leukocyte cell–cell adhesion, T cell activation, regulation of immune system processes, and leukocyte migration (Jambusaria et al., 2020). Hence, in the present study, we used pulmonary ECs in co-culture with iPSCs during differentiation to lung epithelial cells.

Earlier reports suggest that ECs are not required for lung epithelial branching or bud formation (Havrilak et al., 2017; Havrilak and Shannon, 2015), though the pulmonary endothelium is critical for pulmonary epithelial cell differentiation (Yao et al., 2017). However, the pulmonary endothelial plexus is interconnected with lung buds at the embryonic stage. VEGF signaling mediated by endothelial-epithelial crosstalk is known to affect epithelial branching during the embryonic and pseudo-glandular stages (Del Moral et al., 2006; Gebb and Shannon, 2000), suggesting an important role for ECs in early lung development. Here, we showed that co-culture of iPSCs with pulmonary microvascular ECs enhanced the expression of key airway lineage markers during ALI culture. Lung lineage marker NKX2.1 is the earliest progenitor marker of lung epithelial specification (Kimura et al., 1996; Lazzaro et al., 1991). We demonstrated that iPSCs co-cultured with pulmonary microvascular ECs expressed significantly higher levels of *NKX2.1* at most points during the ALI culture period (Fig. 2). As NKX2.1 is also expressed in cells at the early stages of thyroid and ventral forebrain progenitor formation, we further confirmed lung cell development using other airway lineage markers. First, we employed basal cell markers, including KRT5 and TP63. Basal cells are considered relatively undifferentiated, and express KRT5, while TP63 is essential for basal cells with a regenerative capacity in the airway, and also in other organs (Yang et al., 1999). Our results demonstrated that KRT5- and TP63-positive cells were located at the basal surface of the culture adjacent to the basement membrane, and some KRT5-positive cells were also localized around the fenestrations above the basement membrane (Fig. 3C). KRT5-positive cells lacking TP63 are likely the basal cells without progenitor cell function. These cells are common in intact epithelial tissues *in* vivo as seen in other organs (Leir et al., 2020). Our RT-qPCR results demonstrated that co-culture of iPSCs with ECs significantly increased *TP63* expression at the start of ALI culture (weeks 1–3), while enhanced *KRT5* was seen in the later stage of ALI culture (weeks 3–5) (Fig. 3A). We also observed significantly higher of

other non-basal markers in cells with EC co-culture only, within ALI culture weeks 2–1 (including *MUC5AC* at week 3 and 4, *MUC16* at week 2 and 4, *FOXJ1* at week 2 and 3, and *CFTR* at week 2 and 4), indicating that a high regenerative capacity during EC co-culture in the early ALI culture stages may be required to generate more key epithelial cell types. Hematoxylin and eosin staining showed a substantial presence of ciliated epithelial cells under conditions of EC co-culture, which was confirmed by the detection of high levels of *FOXJ1* mRNA. The expression of *SCGB1A1*, *SCGB3A2* and *MUC5B* mRNA was also detected after EC co-cultures, suggesting the presence of club cells among other secretory epithelial cells. Immunostaining with an antibody specific for SCGB1A1 further supported the presence of club cells (Fig. 4F).

In our co-culture system, ECs are seeded inside Matrigel below the basement membrane, causing the ECs to be close to, but not in direct contact with, the iPSC cells, which mimics the location of ECs in the embryonic and pseudo-glandular stages of lung development. As ECs have organ-specific heterogeneity, pulmonary microvascular ECs are most appropriate for use in the study of iPSC differentiation to lung cells. However, we cannot exclude the possibility that human pulmonary microvascular ECs isolated from adult donors might not have all the required EC types that are present in lung tissue during development stages.

In conclusion, the interaction between iPSCs and their microenvironment is important for lung cell differentiation. Appropriate ECM proteins can improve cell growth, and co-culture with ECs during cell differentiation has a significant positive effect on lung cell differentiation, enhancing the expression of key airway lineage marker genes.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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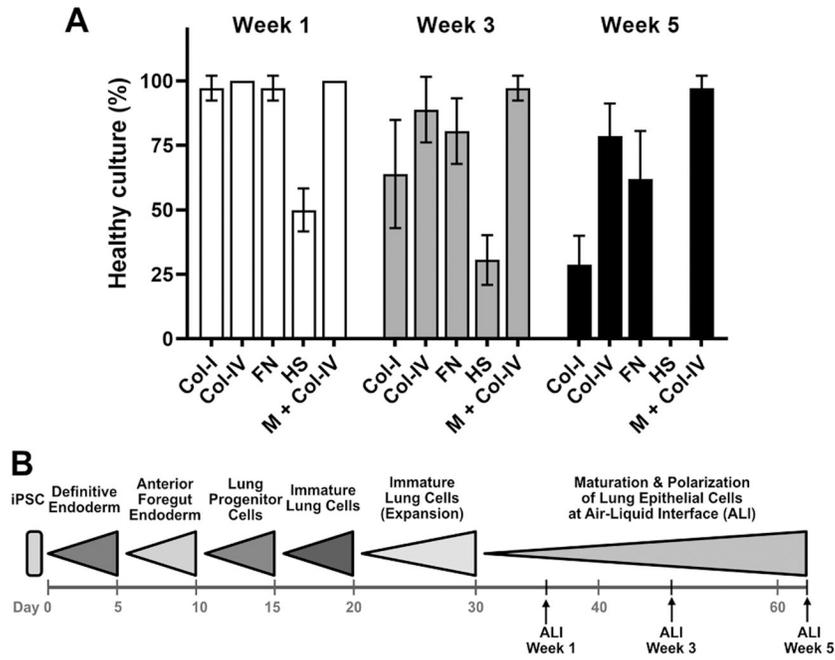
**Fig. 1.**
(A) Success rates of iPSC differentiation to lung epithelial cells on membrane supports coated with different ECM components in air–liquid interface (ALI) culture for 1, 3 and 5 weeks. Collagen I (Col-I), collagen IV (Col-IV), fibronectin (FN), heparan sulfate (HS), and Matrigel + Col-IV (M + Col-IV). (B) Diagram to show differentiation protocol of iPSCs to lung epithelial cell at ALI. Data were the average of three iPSC lines: ND1.4, CWRU205 and GM23476. The experiments were performed at least twice (for each iPSC line, and twelve membrane inserts per iPSC line were used with each matrix protein coating for each line. Healthy cultures were defined based on the intactness of cell layers, as represented by no medium leakage to the upper chamber, and no detachment of or damage to the culture as observed under phase contrast microscope. Success rates = (Number of healthy cultures/Total number of culture) × 100 %.
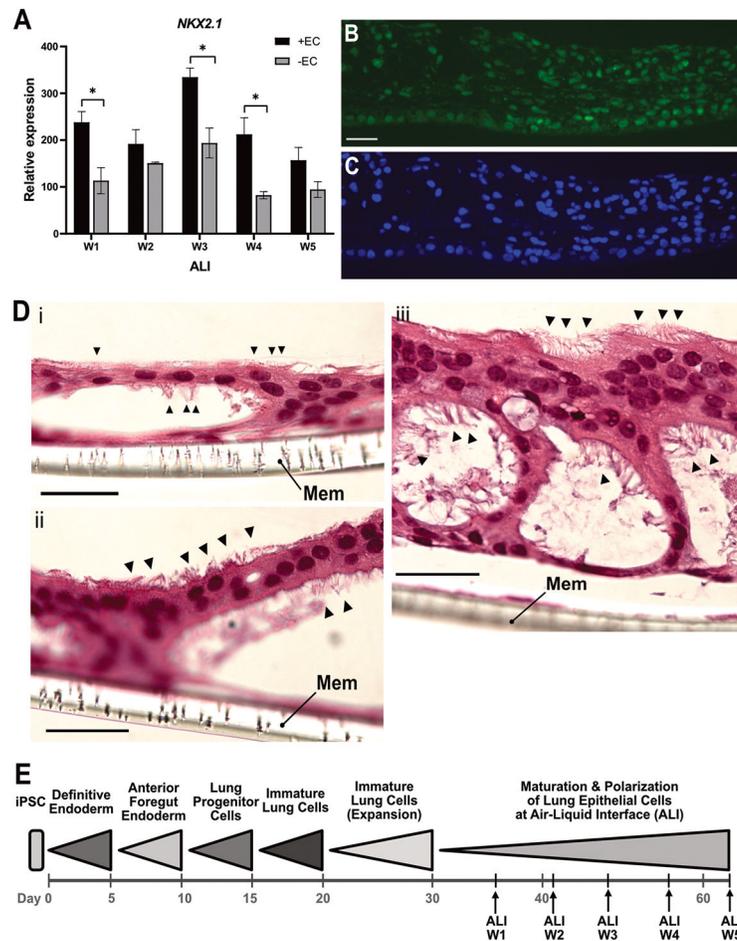
**Fig. 2.**
NKX2.1 expression in iPSC differentiation to lung cells during air–liquid interface (ALI) culture. (A) Effect of endothelial cell co-culture on *NKX2.1* expression during ALI culture at week 1 to week 5. Relative expression was defined as expression of the marker gene normalized to beta-2-microglobulin (β*2M*) and then compared to the average level of expression in 3 human bronchial epithelial (HBE) ALI cultures. (B) Immunostaining of NKX2.1 at ALI culture week 3. (C) DAPI nuclear staining. Scale bar = 40 μm. (D) Representative images of hematoxylin and eosin stain showed ciliated cells (arrowheads) from ALI culture (i) week 1, (ii) week 3 and (iii) week 5. Scale bar = 50 μm. (E) Diagram to show differentiation protocol of iPSCs to lung epithelial cell at ALI. * P < 0.05. (W1-W5: culture under ALI conditions for one to five weeks). Mem: membrane support.
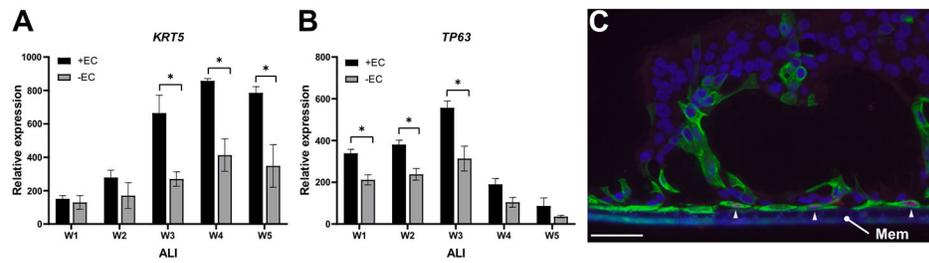
**Fig. 3.**
Basal cell markers of iPSC differentiation to lung epithelial cells in air–liquid interface (ALI) culture. Effect of endothelial cell co-culture on (A) *KRT5* and (B) *TP63* expression during weeks 1 to 5 of ALI culture. Relative expression normalized to the average gene expression of 3 HBE ALI cultures. (C) Immunofluorescence detection of (A) keratin 5 (green) and TP63 (purple; white arrowheads) at week 2 of ALI culture. DAPI nuclear staining (blue). Scale bar = 40 μm. *P < 0.05. (W1-W5: culture under ALI conditions for one to five weeks). Mem: membrane support.
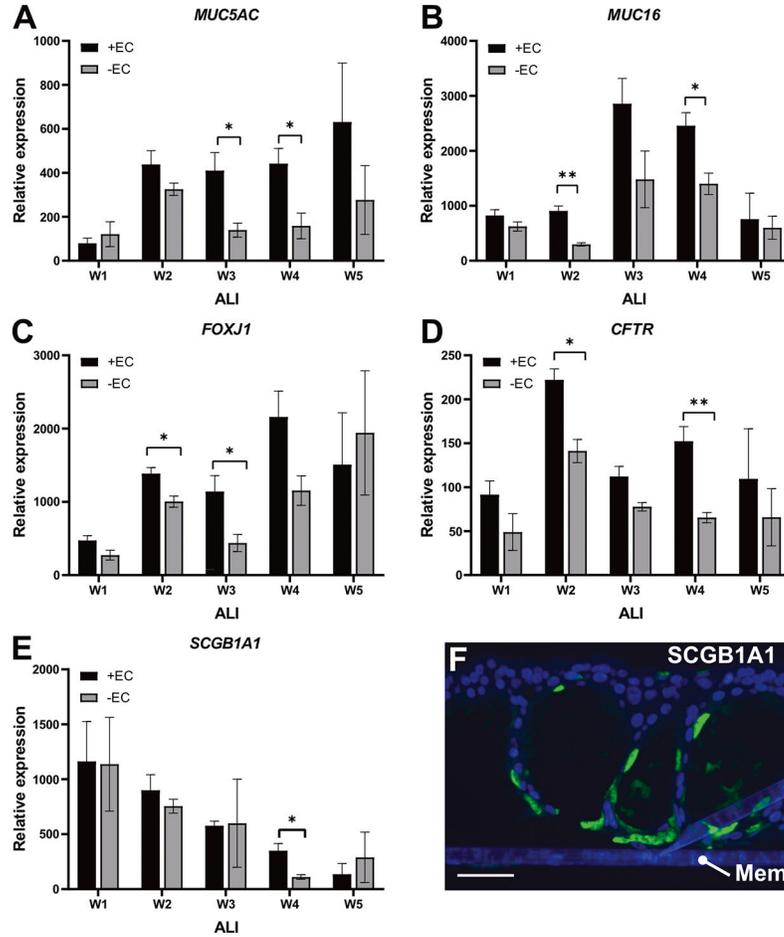
**Fig. 4.**
Marker gene expression in lung epithelial cells in air–liquid interface (ALI) culture. Relative gene expression at weeks 1–5 of ALI culture was normalized as aforementioned. Data were pooled from three individual experiments. (A) *MUC5AC*, (B) *MUC16*, (C) *FOXJ1*, (D) *CFTR*, (E) *SCGB1A1*. (F) SCGB1A1 protein expression (Green) in an ALI week 3 culture and DAPI nuclear staining (blue). Scale bar = 50 μm * P < 0.05. ** P < 0.01. (W1-W5: culture under ALI conditions for one to five weeks). Mem: membrane support.