

# Efficient generation of functional CFTR-expressing airway epithelial cells from human pluripotent stem cells

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**Airway epithelial cells are of great interest for research on lung development, regeneration and disease modeling. This protocol describes how to generate cystic fibrosis (CF) transmembrane conductance regulator protein (CFTR)-expressing airway epithelial cells from human pluripotent stem cells (PSCs). The stepwise approach from PSC culture to differentiation into progenitors and then mature epithelia with apical CFTR activity is outlined. Human PSCs that were inefficient at endoderm differentiation using our previous lung differentiation protocol were able to generate substantial lung progenitor cell populations. Augmented CFTR activity can be observed in all cultures as early as at 35 d of differentiation, and full maturation of the cells in air-liquid interface cultures occurs in <5 weeks. This protocol can be used for drug discovery, tissue regeneration or disease modeling.**

## INTRODUCTION

Discovered in 1998, human embryonic stem cells (ESCs)<sup>1</sup> offer great opportunities for tissue regeneration and basic science research. With the discovery of induced PSCs (iPSCs) by Takahashi and Yamanaka<sup>2</sup> in 2006, it became possible to use patient-specific cells for regenerative medicine, drug discovery and disease modeling. The potential of both human ESCs and iPSCs to generate cell lineages from various tissues has fuelled extensive research in the development of methods to differentiate these cells into the specific cell types of interests. To date, differentiation of human ESCs or iPSCs into cardiac<sup>3</sup>, neuronal<sup>4,5</sup>, retinal<sup>6</sup>, hepatic<sup>7</sup>, intestinal<sup>8</sup>, pancreatic<sup>9</sup> and skin<sup>10</sup> have been established with varying efficiencies in the absence of selection or fractionation. With recent advances in differentiation protocols, mature cell phenotypes capable of exhibiting some functional characteristics of adult cells have been established in liver and endothelial and neuronal models<sup>7,11–13</sup>.

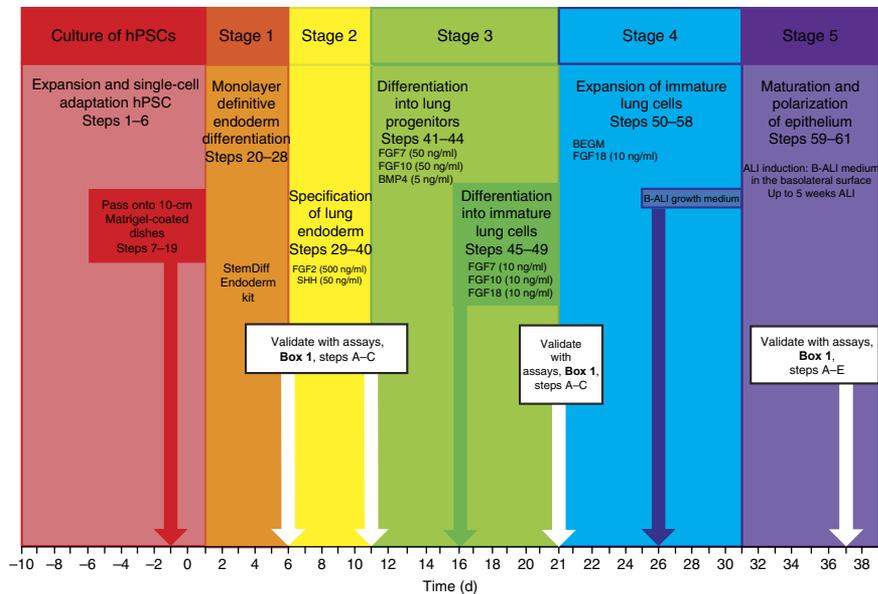
Despite these advances, isolation and expansion of primary epithelial cells remains difficult<sup>14–16</sup>. Most primary cells senesce within a few passages; additionally, these cells can undergo spontaneous transdifferentiation into other epithelial cell types<sup>17</sup> or undergo epithelial-mesenchymal transition owing to the auto-crine production of transforming growth factor- $\beta$  (TGF- $\beta$ ) in culture<sup>18–20</sup>. To circumvent this, many studies have used immortalized primary epithelial cells<sup>21,22</sup>, established cell lines derived from tumors<sup>23</sup> or cells isolated from large animals<sup>24</sup> to study disease mechanisms or regeneration. With the development of iPSCs, tissue-specific cells generated from iPSCs hold great promise for patient-specific disease modeling, drug discovery and personalized medicine.

An example of a disease that could be better studied by improved availability of cell models is CF, a genetically fatal disease with an incidence rate of 1 in 3,500 in Canada<sup>25</sup>. This disease affects the function of the epithelial tissue. Epithelial tissue lines multiple organs throughout the body, including the airways, skin, intestines, pancreatic ducts and reproductive organs. In CF, there

is a lack of ion transport across the airway epithelium, which leads to improper airway fluid balance, mucus thickening and defective mucociliary clearance. This creates a niche for chronic bacterial infections in the airways, which is the main cause of mortality in CF patients. The basic defect is caused by mutations in the *CFTR* gene, which encodes CFTR<sup>26</sup>. The most common CF mutation is caused by a phenylalanine deletion at position 508 (F508del) of the peptide sequence. In 90% of people with CF, F508del is present on at least one allele, whereas in 50% of people with CF the F508del is present on both alleles. However, even with the same CF genotype, modifier genes may account for 54–86% of the variation in CF lung disease<sup>27,28</sup>. The mutant CFTR protein does not fold properly, and it is rapidly degraded instead of trafficked to the cell membrane for chloride transport. To date, CF therapies aim to ameliorate the symptoms of the disease; i.e., they attempt to reduce infection and improve nutritional status. These current therapeutic strategies have succeeded in improving the mean life expectancy from 4 to 50 years of age (CF Canada patient data registry; <http://www.cysticfibrosis.ca/cf-care/cf-registry/>). However, the limited availability of differentiated patient-specific CF lung epithelium remains a major roadblock for the potential development of therapeutic drugs to treat CF.

## Overview of previous methods to derive pulmonary epithelial cells from human PSCs

Early attempts to generate lung epithelial cells from ESCs focused on producing type II alveolar cells that express surfactant protein C (Sftpc)<sup>29–34</sup>. Although these methods were inefficient and heterogeneous in generating lung epithelial cell lineages, these early studies revealed important knowledge about the culture conditions that would eventually be used to generate mature lung epithelia<sup>31,32,35–37</sup>. In 2007, Wang *et al.*<sup>38</sup> demonstrated a method to improve yield and homogeneity of human ESC-derived type II alveolar cells using antibiotic selection in human ESCs containing an Sftpc-driven neomycin selection cassette. Through



**Figure 1** | Timeline of the differentiation protocol procedure.

spontaneous embryoid body-mediated differentiation, Sftpc<sup>+</sup> cells were observed and enriched in the cultures. Lamellar bodies were observed by transmission electron microscopy in these cells. However, no functional characterization of these cells was performed to determine whether these were bona fide type II alveolar cells.

Recently, there have been major breakthroughs in the ability to generate mature lung epithelial cells from human ESCs<sup>39–41</sup>. On the basis of mouse lung developmental studies, stepwise differentiations of human ESCs (hESCs) to lung epithelial cells have yielded more extensive lung epithelial cell phenotypes than previously published. These cells include type II alveolar cells<sup>30,33</sup> of the respiratory epithelium, Clara cells<sup>37</sup> of the conducting airways, ciliated cells and basal cells of the proximal airways<sup>39–41</sup>. Furthermore, improved methods have generated cells that have demonstrated efficacy in proof-of-concept patient iPSC-derived models for drug screens, disease modeling *in vitro* and tissue regeneration<sup>39,41,42</sup>. The robust and renewable source of patient-specific lung epithelial cells holds great promise for their future use in preclinical studies and regenerative medicine.

We have previously shown the differentiation of hESCs into ciliated epithelial cells of the proximal airways that express functional CFTR protein<sup>39</sup>. These cells could be used to study the effects of small-molecule compounds on CFTR regulation from CF patient iPSC-derived airway epithelial cells. Here we describe a modified robust protocol for the generation of CFTR-expressing proximal airway epithelia from human ESCs or iPSCs.

**Figure 1** illustrates an overview of the entire procedure. To date, three human ESC lines (CA1, H9 and HES2), as well as two CF-iPSC lines, were successfully differentiated using this modified protocol. By using our previous protocol<sup>39</sup>, CFTR function could be detected in ~25% of the CA1 cultures with each differentiation sets. For the H9 cultures, on rare occasions we were able to detect CFTR function (often more robust than CA1); however, the detection of function was often inconsistent with each differentiation sets. hESCs often display variable efficiency in the

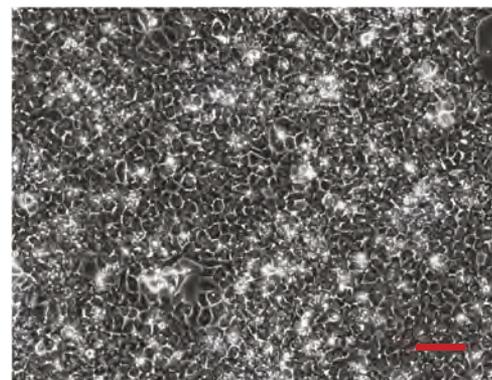
establishment of robust definitive endoderm (DE). We feel that this DE variability was partly responsible for the differences of CFTR functions that we observed between each differentiation cultures. With the modified protocol described here, robust DE cells are generated with H9 hESCs, and CFTR function can be measured reproducibly in all CA1 hESC cultures with every differentiation sets. Furthermore, the level of CFTR function in CA1 is substantially augmented compared with our previous protocol.

**Experimental design**

The following procedure describes the preparation of the cells for the differentiation and the entire process of differentiation. In the differentiation part of the procedure, days are numbered, with day 1 being the first day of differentiation. Characterization of the cell types formed at each stage can be performed by FACS,

immunofluorescence staining or RT-PCR. We recommend that differentiation cultures be doubled at each stage if you are performing such a characterization.

**Routine culture stage: culture, expansion and adaptation of human PSCs to single-cell culture (Steps 1–19)** Unlike mouse ESCs, human PSCs are traditionally passaged as small clumps of cells rather than as a single-cell suspension. However, to improve differentiation efficiency and differentiate cells as a monolayer, hPSCs must be adapted to a single-cell culture system. This is achieved by the slow introduction of trypsin-mediated passaging to the culture method. Human PSCs are typically maintained and expanded on mouse embryonic fibroblasts (MEFs) with a split ratio of 1:6 weekly in feeder-dependent conditions in knockout DMEM medium containing knockout serum replacement (20%) and basic fibroblast growth factor 2 (FGF2; 10–20 ng/ml). In place of collagenase to split the cells, use TrypLE (a more gentle form of trypsin). Optimal treatment time must be determined for each line to yield viable single cells that can be expanded in culture, as



**Figure 2** | Photomicrograph image of stage 1 DE cells after 4 d of StemDiff DE treatment. Scale bar, 100 μm.

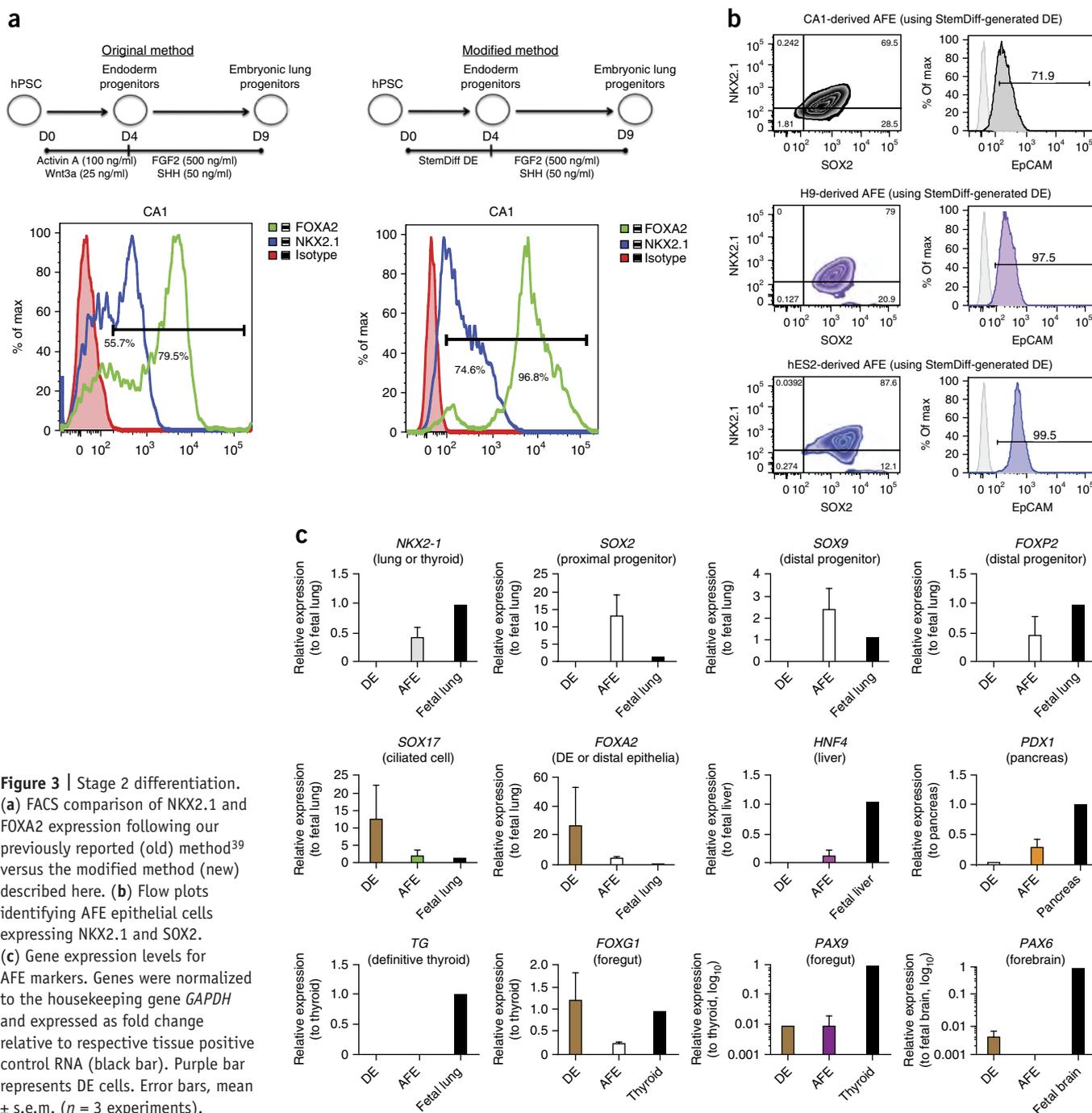
there is line-to-line variability in the ability to adapt to trypsin-mediated passaging. This protocol is optimized for the human ESC line CA1. Expansion of hESCs or hiPSCs and adaptation to single-cell passaging can take up to 8 weeks.

**Stage 1: differentiation of human PSCs into DE progenitors (Steps 20–28)** The first stage of differentiation is to establish a pure population of DE cells. A high yield of DE progenitors (Fig. 2) will improve the efficiency of later differentiation into specialized endoderm derivatives. Since our previous publication<sup>39</sup>, we have switched to using StemDiff definitive endoderm kit (Stem Cell Technologies) and found that this kit was highly efficient at generating >95% DE (CXCR4<sup>+</sup>FOXA2<sup>+</sup>) cells in most of our PSC lines. Furthermore, cell lines that were inconsistent at

generating endoderm previously, such as the hESC line H9, can now differentiate efficiently and robustly using this kit. One kit can be used for two differentiations (two 10-cm dishes).

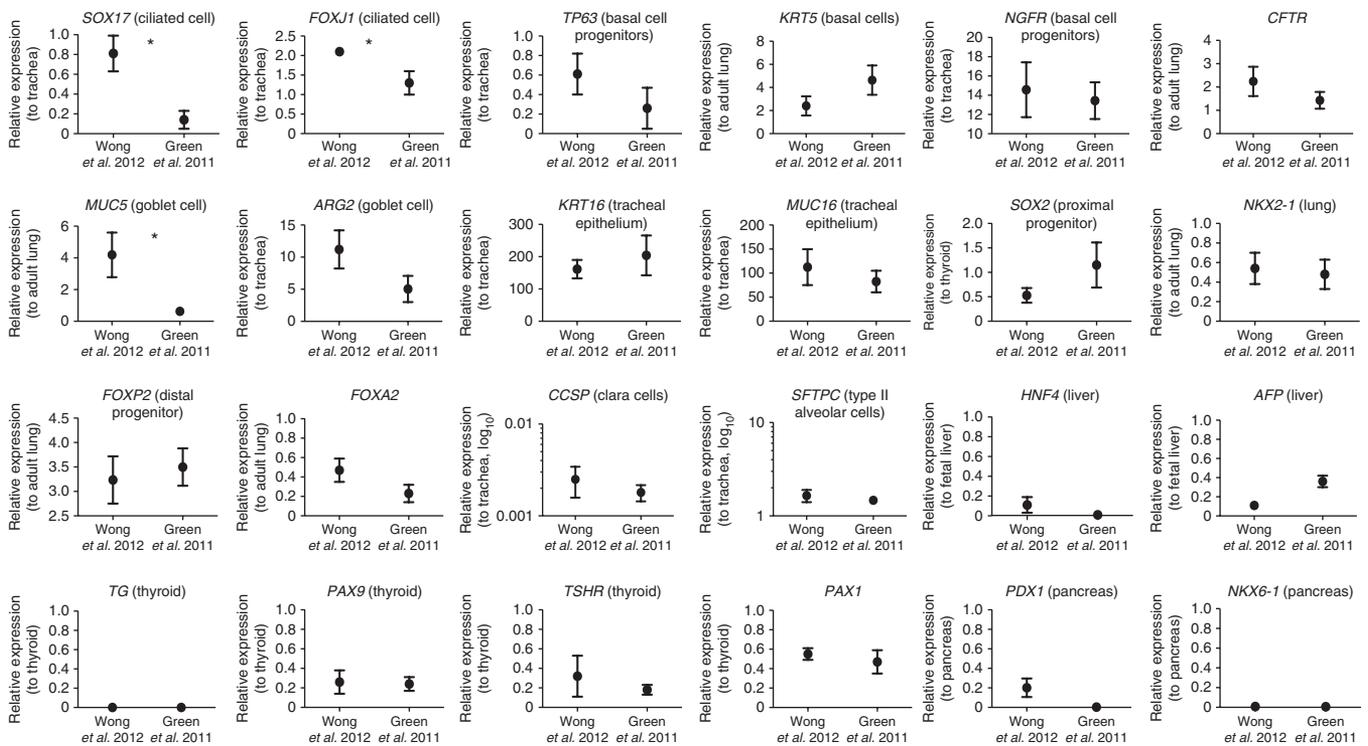
At the end of DE generation (Step 28), assay for DE protein expression by performing FACS for the combination of CXCR4, CD117 (cKit) and SOX17, FOXA2. Real-time RT-PCR can also be used to assay for DE gene upregulation compared with undifferentiated hESCs.

**Stage 2: differentiation of DE progenitors to embryonic lung progenitors (Steps 29–40)** After DE generation, cells are directed to form embryonic lung progenitors using sonic hedgehog (SHH) and high concentrations of FGF2. Studies have shown that cardiac mesoderm secretes FGF2, and depending on concentration



**Figure 3 | Stage 2 differentiation.** (a) FACS comparison of NKX2.1 and FOXA2 expression following our previously reported (old) method<sup>39</sup> versus the modified method (new) described here. (b) Flow plots identifying AFE epithelial cells expressing NKX2.1 and SOX2. (c) Gene expression levels for AFE markers. Genes were normalized to the housekeeping gene *GAPDH* and expressed as fold change relative to respective tissue positive control RNA (black bar). Purple bar represents DE cells. Error bars, mean ± s.e.m. (*n* = 3 experiments).

# PROTOCOL

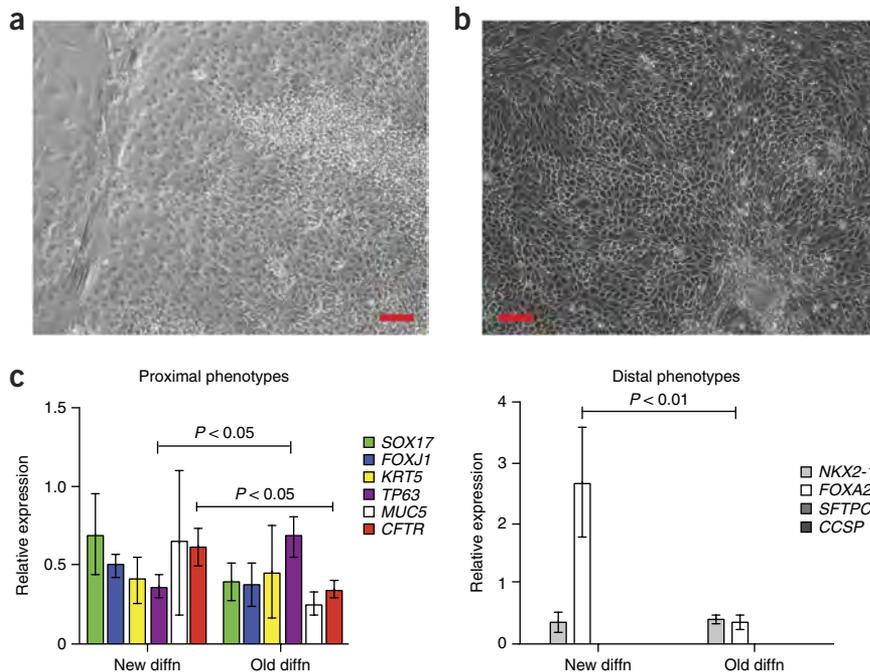


**Figure 4** | Comparison of gene expression levels associated with lung, liver, pancreas and thyroid after 5 weeks of ALI using our previous protocol with FGF2 and SHH to induce anterior foregut and lung specification<sup>39</sup> versus TGF- $\beta$  and BMP4 inhibition<sup>46</sup>. Genes were normalized to the housekeeping gene *GAPDH* and expressed as fold change relative to respective tissue positive control RNA. Error bars, mean  $\pm$  s.e.m. ( $n = 3$  experiments). Asterisk (\*) denotes statistical significance;  $P < 0.05$ .

it drives specification of the foregut endoderm lineages<sup>36</sup>. Owing to proximity *in vivo*, a high concentration of FGF2 drives lung fate, whereas a low concentration of FGF2 drives liver fate and intermediate concentrations promote pancreatic development<sup>43</sup>. Similarly, SHH has been shown to have an important role in lung development, as it is highly expressed in the distal lung bud where

it controls epithelial proliferation and mesenchymal cell growth during branching morphogenesis<sup>44</sup>. In addition, SHH has been shown to suppress pancreatic lineage development<sup>45</sup>. Therefore, the combination of SHH and high concentrations of FGF2 promotes lung specification and suppresses pancreatic development. Improving DE generation in stage 1 augments the number of

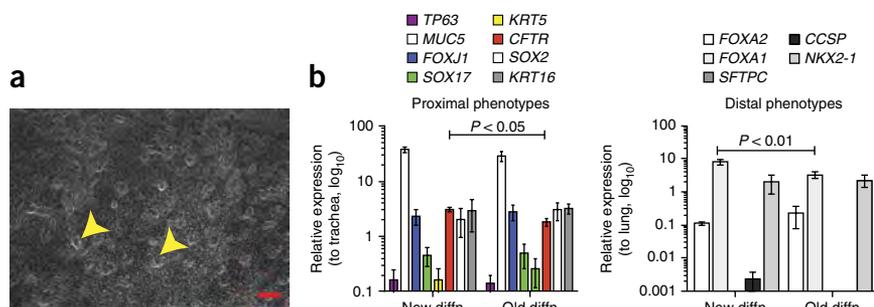
NKX2-1<sup>+</sup> cells after stage 2 (Fig. 3a,b). However, it is important to note that this differentiation protocol still does not yield 100% pure lung progenitors (Fig 3c). Liver- and pancreas-specific genes continue to be detected, although in a lower proportion of cells compared with that seen using our previous method<sup>39</sup>. Morphologically, the monolayer of cells appears to be similar to stage 1 cells forming tight cell-to-cell contact (data not shown).



**Figure 5** | Stage 3 differentiation. (a,b) Representative stage 3a cells (a) and stage 3b cells (b). Cells were cultured on collagen-coated plastic culture plates to capture better morphology. (c) Average gene expression level comparing new (left) and old (right) differentiation methods. Genes were normalized to the housekeeping gene *GAPDH* and expressed as fold change relative to control tissue RNA (lung for distal markers and trachea for proximal markers). Error bars, mean  $\pm$  s.e.m. ( $n = 3$  experiments). Scale bars, 100  $\mu$ m.

**Figure 6** | Stage 4 differentiation.

(a) Representative image of stage 4 cells. Yellow arrowhead points to areas of stacked cells. (b) Average gene expression level of stage 4 cells comparing new (left) and old (right) differentiation methods. Genes were normalized to the housekeeping gene *GAPDH* and expressed as fold change relative to control tissue RNA (lung for distal markers and trachea for proximal markers). Error bars, mean  $\pm$  s.e.m. ( $n = 3$  experiments). Scale bar, 100  $\mu$ m.

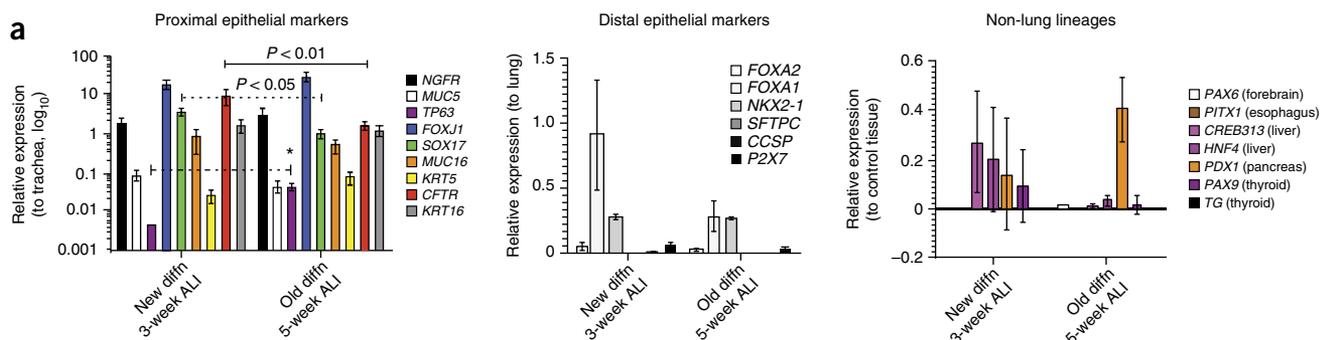


At the end of stage 2 (Step 40), assay for anterior foregut endoderm establishment, specifically lung endoderm, by FACS for the combination of NKX2-1 and SOX2, as well as NKX2-1 and FOXA2. Real-time RT-PCR can also be used to assay for upregulation of anterior foregut endoderm genes (Fig. 3c). If available, fetal tissue cDNA-positive controls (see list of positive human RNA controls in the MATERIALS section) should be used to test the primer efficiency and to compare the level of foregut marker expression. DE cells should be used as ‘negative’ control. Lung endoderm genes should be comparatively lower/not detectable in DE cells.

**Stage 3: generation of immature lung cells (Steps 41–49)** The next stage involves directing the cells toward an immature lung cell fate

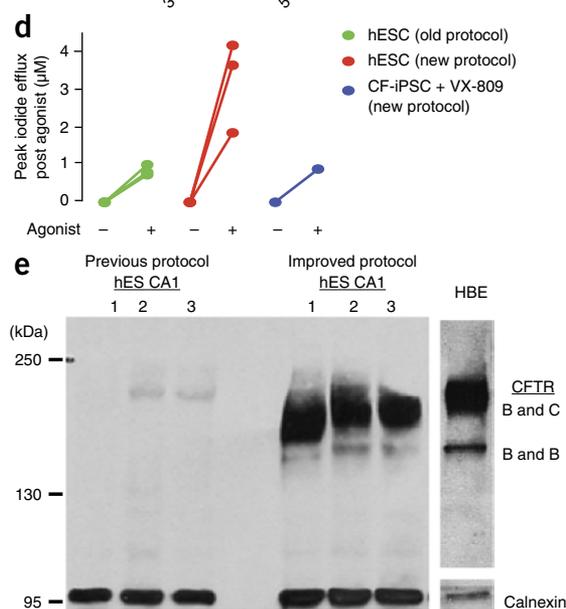
using the combination of epithelial morphogens FGF7, FGF10, BMP4 and FGF18 (ref. 39). Previous methods have described a brief inhibition of the bone morphogenetic protein (BMP)/TGF- $\beta$ /Wnt pathways using small molecules to induce ‘anteriorization’ of the endoderm, followed by treatment with ‘ventralizing’ factors WNT, FGF10, FGF7, BMP and RA to generate embryonic lung cells<sup>46</sup>. We have compared the current protocol and that used in Green *et al.*<sup>46</sup> and found that, although most genes are expressed at comparable levels in the final stage of differentiation after 5 weeks of air-liquid interphase (ALI), our previously published method<sup>39</sup> generated higher expression of ciliated and mucus-producing cells (Fig. 4).

At the end of stage 3 (Step 49), cell morphology appears as an epithelial monolayer (Fig. 5a,b). Lung epithelial gene expression



**Figure 7** | Stage 5 differentiation.

(a) Average gene expression level of stage 5 cells from three independent hESC lines comparing new (left) and old (right) differentiation methods. Genes were normalized to the housekeeping gene *GAPDH* and expressed as fold change relative to respective control tissue RNA. Error bars, mean  $\pm$  s.e.m. ( $n = 3$  experiments). (b) Flow cytometric analysis of panKRT, CFTR and NKX2-1 (all blue histograms) demonstrating that a large proportion of the cells are epithelial CFTR-expressing cells. Pink histogram represents isotype staining. (c) Representative cross-section of an immunofluorescence staining of panCK (red) and CFTR (green) of 30-week ALI-differentiated hESCs (top) and immunofluorescence staining of an ALI culture for ZO1 (red) and panCK (green) (bottom). Scale bars, 22  $\mu$ m. (d) Iodide efflux function comparing old (green), new (red) differentiation culture methods of hESC CA1 and F508del mutant line GM00997 CFiPS-derived cells treated with VX-809 (blue). (e) Western blot for mature band C and immature band B form of CFTR protein. Calnexin was used as loading protein control. Human bronchial epithelial (HBE) cells were used as positive control line for CFTR protein level. Asterisk denotes statistical significance;  $P < 0.01$ .



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can be determined by FACS or real-time quantitative RT-PCR (qRT-PCR; Fig. 5c). If available, isolated primary epithelial cell from human lung tissue should be used as a positive control for FACS; however, these may be difficult to obtain. Alternatively, primary human bronchial epithelial cells can be purchased from Lonza and differentiated under ALI conditions. These cells can be used as controls for FACS or immunocytochemistry. For real-time qRT-PCR, cDNA from RNA tissue samples should be used as positive controls and set as the 'standard' level to achieve for the differentiations. As it can be unclear where the tissues were resected to establish the RNA samples, there could be lot-to-lot variability when obtaining the RNA. It is thus best to use the same lot sample as the control for the entire differentiation process.

**Stage 4: expansion of immature lung cells (Steps 50–58)** This stage allows for the expansion of lung epithelial progenitors, using bronchial epithelial cell growth medium (BEGM), a commercially available medium to support lung epithelial cells. At this stage, the cells can be highly proliferative and medium changes may need to be more frequent than described in this protocol. Expand the cells at a 1:2–1:3 split ratio for no more than three passages. The morphology of the cells should be a cuboidal epithelial layer.

At the end of stage 3 (Step 58), the cells may start to grow on top of one another, forming 3D-like structures (Fig. 6a) that appear pseudostratified when they are cross-sectioned. Determine lung epithelial gene expression by FACS or real-time qRT-PCR (Fig. 6b). Positive controls used for this stage are the same as for those used for stage 3.

**Stage 5: maturation and polarization of lung epithelia (Steps 59–61)** This final stage involves exposing the cultures to air. Culturing the cells at the ALI induces polarization and maturation of the cells. Cells can be maintained in ALI for at least 5 weeks. With the use of the StemDiff DE kit at stage 1, the cells appear to mature and polarize much faster than was reported in our previous publication<sup>39</sup>. Mucus can be observed on the apical side of the Transwell as early as 1 week after ALI culture. As a result, changing of the medium on cultures becomes increasingly difficult, as removal of the mucus can often result in peeling off of the epithelium from the membrane. Therefore, it is crucial to be extra careful when you are removing the mucus and liquid from the apical side of the epithelium. The cells do not proliferate very well at this stage. Beating cilia should be observed between 3 and 5 weeks of ALI. On rare occasions, cilia can be observed earlier.

At the end of stage 5 (Step 61), assay for lung epithelial and nonlung gene expressions by real-time qRT-PCR (Fig. 7a), FACS (Fig. 7b) or immunofluorescence staining (Fig. 7c), as well as CFTR function (Fig. 7d) and of the relative expression of the mature fully glycosylated form of the CFTR protein (Fig. 7e) by western blotting. For FACS, immunofluorescence and real-time qRT-PCR, use the same controls as described in Stage 3. For western blot analysis, Caco-2 cells, a human intestinal epithelial cell line that expresses CFTR and therefore expresses an abundant level of CFTR protein, can be used as a positive control. For CFTR function, use DMSO (unstimulated control) to determine baseline CFTR function. Caco-2 should be used as a positive control for functional analysis as well.

## MATERIALS

### REAGENTS

- Human ESC lines HES2 and H9 (Wicell) and CA1 **! CAUTION** Take precaution when working with human samples. They should be handled in a biological safety cabinet. **! CAUTION** All work with human cell lines must adhere to all relevant institutional and governmental regulations.
- Human induced pluripotent stem cell lines GM00997 Line no. 2 (ref. 39) and GM04320 Line no. 2 (original cell source from Coriell) **! CAUTION** Take precaution when you are working with human samples. They should be handled in a biosafety cabinet.
- FGF2 (Preprotech, cat. no. AF-100-18B)
- SHH (Cedarlanes, cat. no. CLCYT676-2)
- BMP4 (R&D Systems, cat. no. 314-BP)
- FGF10 (R&D Systems, cat. no. 345-FG)
- FGF7 (R&D Systems, cat. no. 251-KG)
- FGF18 (Sigma-Aldrich, cat. no. F7301)
- Wnt3a (R&D Systems, cat. no. 5036-WN)
- StemDiff DE kit (Stem Cell Technologies, cat. no. 05110)
- Inactivated MEFs (E12.5 embryos)
- PBS +Ca<sup>2+</sup>+Mg<sup>2+</sup> (Life Technologies, cat. no. 14200075)
- PBS –Ca<sup>2+</sup>–Mg<sup>2+</sup> (Gibco, cat. no. 14190-144-250)
- TrypLE (Life Technologies, cat. no. 12604013)
- Collagenase type IV (Life Technologies, cat. no. 17104-019)
- Y-27632 (Rho-associated kinase (ROCK) inhibitor, Stem Cell Technologies, cat. no. 72302)
- Human placental collagen type IV (Sigma-Aldrich, cat. no. C7521)  
**▲ CRITICAL** Cell attachment is sensitive to batch-to-batch variability. Test batches and order sufficient vials of the same batch for the entire experimental procedure.
- mTESR1 (Stem Cell Technologies, cat. no. 05850) **▲ CRITICAL** Growth of pluripotent cells seems to be sensitive to mTESR1 batches. Test the batch and order a large stock of the same batch for the experiment.
- KnockOut DMEM (Gibco, cat. no. 10829-018)
- KnockOut serum replacement (Gibco, cat. no. 10828-028)
- DMEM/F12 (Gibco, cat. no. 1130-032)
- DMEM (Gibco, cat. no. 11960-044)
- Penicillin-streptomycin (Gibco, cat. no. 15140-122)
- Matrigel, growth factor reduced (BD, cat. no. 354277)
- β-Mercaptoethanol (Gibco, cat. no. 21985-023) **! CAUTION** β-mercaptoethanol is very hazardous in case of skin contact or ingestion. Please use this reagent in a biosafety cabinet and follow the manufacturers' instructions.
- Glutamax (Gibco, cat. no. 35050-061)
- Mono-thioglycerol (MTG, Sigma-Aldrich, cat. no. M6145)
- Nonessential amino acid (Gibco, cat. no. 11140-050)
- RNeasy mini kit (Qiagen, cat. no. 74106)
- B-ALI bullet kit (Lonza, cat. no. 00193514) **▲ CRITICAL** There are competing media that support epithelial cell growth and differentiation in the air-liquid interface. This kit has been most reproducible for ALI establishment.
- BEGM bullet kit (Lonza, cat. no. CC-3170)
- Anti-TTF1 1:100 (Abcam, cat. no. ab76013)
- Anti-TTF1 1:200 (Cedarlanes, cat. no. CLS3686905)
- Anti-FOXA2 1:500 (Abcam, cat. no. ab40874)
- Anti-EpCAM 1:100 (Abcam, cat. no. ab20160)
- Anti-pan-cytokeratin 1:500 (Abcam, cat. no. ab80826)
- Anti-p63 1:100 (Abcam, cat. no. ab32353)
- Anti-FOXJ1 1:100 (Abcam, cat. no. ab40869)
- Anti-LHS28 1:200 (Abcam, cat. no. ab14373)
- Anti-MUC5ac 1:200 (Abcam, cat. no. ab3649)
- Anti-MUC16 1:200 (Abcam, cat. no. ab1107)
- Anti-SOX17 1:100 (R&D Systems, cat. no. MAB1924)
- Anti-SOX17-APC 1:50 (R&D Systems, cat. no. IC1924A)
- Anti-SOX2 1:200 (GeneTex, cat. no. GTX101507)
- Anti-TRA1-60 1:100 (Zymed, cat. no. 41-1000)
- Anti-TRA1-81 1:100 (Zymed, cat. no. 41-1100)
- Anti-ZO1 1:100 (Invitrogen, cat. no. 33-9100)
- Anti-ZO1 1:100 (Abcam, cat. no. ab59720)

- Anti-βIV-tubulin 1:200 (Abcam, cat. no. ab15568)
- Anti-CD184-PECy7 1:200 (BD, cat. no. 560669)
- Anti-CD117-FITC 1:100 (BD, cat. no. 553354)
- Anti-CFTR 1:100 (Millipore, cat. no. MAB3484)
- Anti-CFTR 1:50 (R&D Systems, cat. no. MAB1660)
- Anti-CFTR 1:500 (courtesy of J.R. Riordan, no. 450)
- Anti-CFTR 1:1,000 (courtesy of J.R. Riordan, no. 596)
- Anti-CFTR 1:500 (courtesy of J.R. Riordan, no. 660)
- Anti-cytokeratin-16 1:200 (Novus Biologicals, cat. no. NB110-62105)
- Goat anti-mouse IgG (H+L) Alexa Fluor 488 1:500 (Invitrogen, cat. no. A-11001)
- Goat anti-rabbit IgG (H+L) Alexa Fluor 488 1:500 (Invitrogen, cat. no. A-11008)
- Goat anti-mouse IgG (H+L) Alexa Fluor 532 1:500 (Invitrogen, cat. no. A-11002)
- Goat anti-rabbit IgG (H+L) Alexa Fluor 532 1:500 (Invitrogen, cat. no. A-11009)
- Goat anti-mouse IgG (H+L) Alexa Fluor 633 1:500 (Invitrogen, cat. no. A-21052)
- Goat anti-rabbit IgG (H+L) Alexa Fluor 633 1:500 (Invitrogen, cat. no. A-21071)
- Fluorescence mounting medium (Dako, cat. no. S3023)
- Ultrapure distilled water (Invitrogen, cat. no. 10977-015)
- BSA (Sigma-Aldrich, cat. no. A1470)
- FBS (Gibco, cat. no. 12483-020)
- DMSO (Sigma-Aldrich, cat. no. D2650)
- Triton X-100 (Sigma-Aldrich, cat. no. T8787)
- DAPI (Molecular Probes, cat. no. D3571)
- Paraformaldehyde (PFA; Merck, cat. no. 1.04005.1000) **! CAUTION** PFA has potential hazardous effects when in direct contact. When you are using this chemical, work should be performed in a fume hood.
- Gelatin from porcine skin, type A (Sigma-Aldrich, cat. no. G1890)
- Trypsin, 0.25% (wt/vol) (Gibco, cat. no. 25200-072)
- Gentle cell dissociation reagent (Stem Cell Technologies, cat. no. 07174)
- SYBR Green I master (Roche, cat. no. 04 997 352 001)
- BD Cytotfix/Cytoperm solutions (10×, BD Biosciences, cat. no. 554714)
- Mitomycin C (MMC; Sigma-Aldrich, cat. no. M0503) **! CAUTION** Mitomycin C is a carcinogenic and mutagenic substance. Use gloves when you are handling this substance, and open it in a biosafety cabinet. Minimize overexposure.
- Normal goat serum (Life Technologies, cat. no. PCN5000)
- Methanol (Fischer Scientific, cat. no. BPA412-1) **! CAUTION** Mutagenic and potentially teratogenic substance. Use gloves when you are handling it, and use it in the fume hood. Minimize overexposure.
- Anhydrous alcohol (VWR, cat. no. CA48218-640)
- Superscript II (Invitrogen, cat. no. 18064-014)
- Oligo dT (Invitrogen, cat. no. 18418-012)
- dNTPs (Invitrogen, cat. no. 10297-018)
- Amiloride (Sigma-Aldrich, cat. no. A7410) **! CAUTION** Amiloride may cause skin, eye and respiratory irritations. Wear protective eye shields, dust mask and gloves. Minimize overexposure.
- Forskolin (Sigma-Aldrich, cat. no. F6886) **! CAUTION** Forskolin may cause skin, eye and respiratory irritations. Minimize overexposure.
- IBMX (Sigma-Aldrich, cat. no. I5879) **! CAUTION** IBMX may cause skin, eye and respiratory irritations. Minimize overexposure.
- Genistein (Sigma-Aldrich, cat. no. G6649) **! CAUTION** Genistein may cause reproductive defects. Weigh the powder in the fume hood and minimize overexposure.
- VX-809 (Selleckchem, cat. no. S1565)
- VX-770 (Selleckchem, cat. no. S1144)
- Protease K inhibitor (Amresco, cat. no. M221)
- SDS (Wisent, cat. no. 800-100-EG) **! CAUTION** SDS is slightly hazardous. Use it in the fume hood and minimize overexposure.
- 10× Tris-glycine-SDS buffer (Wisent, cat. no. 880-570-LL)
- Tween 20 (Sigma-Aldrich, cat. no. P2287)
- Sodium iodide (Sigma-Aldrich, cat. no. 217638)
- HEPES (Sigma-Aldrich, cat. no. H3375)
- Calcium nitrate (Sigma-Aldrich, cat. no. C2786)
- Potassium nitrate (Sigma-Aldrich, cat. no. P6030)
- Glucose (Sigma-Aldrich, cat. no. G7528)
- Trizma (Bioshop, cat. no. TRS001)
- DTT (Sigma-Aldrich, cat. no. D0632)

- Sodium chloride (Bioshop, cat. no. SOD004)
- Sodium phosphate dibasic (Bioshop, cat. no. SPD307)

**EQUIPMENT**

- Transwells, six wells (Costar, cat. no. 3460)
- Filters, 0.2 mm (Nalgene, cat. no. 73520-994)
- Culture plates, six wells (Nunc, cat. no. 140685)
- Cell scraper (Sarstedt, cat. no. 83.1830)
- CO<sub>2</sub> cell culture incubator (Hera Cell 150, Thermo Scientific)
- Sterile biosafety cabinet (Labconco Class II Biosafety Cabinet, Logic)
- Leica microscope system (Leica, DFC340FX)
- Tubes, 50-ml polypropylene tube (BD Falcon, cat. no. 352070)
- Tubes, 15-ml polypropylene tube (Sarstedt, cat. no. 62.554.205)
- Sterile serological plastic pipettes (Sarstedt; 5 ml, cat. no. 86.1253.001; 10 ml, cat. no. 86.1254.001; and 25 ml, cat. no. 86.1685.001)
- Filter tips (Corning; 20 µl, cat. no. 4821; VWR; 200 µl, cat. no. 89174-526; and 1,000 µl, cat. no. 83007-386)
- Pipetman starter kit (P20, P200, P1000; VWR)
- Pipette-aid XL (Drummond, cat. no. 4-000-085)
- Virox intervention wipes (Accel)
- Tissue culture Petri dish, 10 cm (Nunc, cat. no. 172958)
- 5-ml Polystyrene round-bottom tube with a cell strainer (BD Biosciences, cat. no. 352235)
- Allegra X-22R centrifuge (Beckman Coulter)
- Glass coverslips (VWR, cat. no. 48393.060)
- Glass microscope slides (Superfrost Plus, VWR, cat. no. 48311-703)
- Hemocytometer (Haussen Scientific, cat. no. 3200)
- Becton Dickinson (BD) analyzer (BD LSRII)
- Quorum Spinning Disk confocal microscope (Olympus IX81)
- Eppendorf tubes, 1.2 ml (Axygen, cat. no. MCT-150-C)
- Cryovials, 1.2 ml (Nalgene, cat. no. 66008-706)
- Square-bottom plastic bottle, 250 ml (Nalgene, cat. no. 2019-0250)
- Square-bottom plastic bottle, 125 ml (Nalgene, cat. no. 2019-0125)
- Freezer boxes (Nalgene, cat. no. CS509X10)
- Parafilm (VWR, cat. no. 52858-000)
- Light Cycler LC480 (Roche)
- Chamber slides, eight well (Thermo Scientific, cat. no. 154534)
- NanoDrop 2000c (Thermo Scientific)
- Thermal Cycler (MJ Research PTC-2000)
- PCR tubes, 0.5 ml (Axygen, cat. no. 321-10-061)
- PCR plate sealing tape (Sarstedt, cat. no. 95.1994)
- PCR plate, 96 well (Sarstedt, cat. no. 72.1982.202)
- Iodide sensitive combination microelectrode (Lazar Research Laboratories, cat. no. ISM-1461C)
- Axon Digidata data acquisition system (Molecular Devices, cat. no. 1230A)
- pCLAMP, Clampex 8 software (Molecular Devices)
- Beckman Spinchron DLX centrifuge (Beckman Coulter)

**REAGENT SETUP**

**Collagen stock solution (600 µg/ml)** Dissolve 15 mg of collagen type IV with 25 ml of deionized water and 50 µl of glacial acetic acid in a beaker. Cover the holding beaker with Parafilm and stir it moderately at room temperature (22–25 °C) until the collagen is dissolved, which takes ~15–20 min. Filter-sterilize the solution with a 0.2-µm-pore filter. Prepare 1-ml aliquots and freeze them at –20 °C for up to 6 months.

**▲ CRITICAL** It is best to prescreen a lot before buying a large quantity. Lots can vary in terms of potency of cell attachment and possible toxicity to cells.

**Gelatin solution, 0.1% (wt/vol)** Dissolve 100 mg of gelatin in 100 ml of PBS (+Ca<sup>2+</sup>+Mg<sup>2+</sup>). Filter-sterilize the solution using a 0.22-µm membrane filter. Store the solution at 4 °C for up to 3 months.

**FACS buffer** Dissolve 5 g of BSA in 250 ml of PBS. Sterilize the buffer using a Stericup filter (0.22 µm), and store it at 4 °C for up to 3 months.

**BSA, 1% (wt/vol) in PBS** Dissolve 1 g of BSA in 100 ml of PBS. Sterilize the solution by using a 0.22-µm membrane filter, and store it at 4 °C for up to 4 weeks.

**PFA, 4% (vol/vol)** Dilute 10% (vol/vol) PFA in PBS by mixing 20 ml of 10% PFA with 30 ml of PBS. This solution can be stored at 4 °C for up to 2 weeks.

**! CAUTION** This should be performed in a fume hood.

**PFA, 1% (vol/vol)** Dilute 10% (vol/vol) PFA in PBS by mixing 5 ml of 10% PFA with 45 ml of PBS. This solution can be stored at 4 °C for up to 2 weeks.

**! CAUTION** This should be performed in a fume hood.

## PROTOCOL

**0.25% (vol/vol) Triton X-100 in PBS** Add 1.25 ml of Triton X-100 to 500 ml of PBS. Sterilize the solution by filtrating using a 0.22- $\mu$ m membrane filter, and store it at room temperature for up to 1 year.

**BMP4 (25  $\mu$ g/ml stock solution)** Reconstitute the contents of the vial to a concentration of 25  $\mu$ g/ml in sterile 4 mM HCl containing 0.1% (wt/vol) BSA. Prepare aliquots and store them at  $-80^{\circ}\text{C}$  for up to 1 year.

**FGF2 (50  $\mu$ g/ml stock solution)** Reconstitute the contents of the vial to a concentration of 50  $\mu$ g/ml in PBS containing 1% (wt/vol) BSA. Prepare aliquots and store them at  $-80^{\circ}\text{C}$  for up to 1 year.

**Activin A (25  $\mu$ g/ml stock solution)** Reconstitute the contents of the vial to a concentration of 25  $\mu$ g/ml in sterile PBS containing 1% (wt/vol) BSA. Prepare aliquots and store them at  $-80^{\circ}\text{C}$  for up to 1 year.

**FGF10 (100  $\mu$ g/ml stock solution)** Reconstitute the contents of the vial to a concentration of 100  $\mu$ g/ml in sterile PBS containing 1% (wt/vol) BSA. Prepare aliquots and store them at  $-80^{\circ}\text{C}$  for up to 1 year.

**FGF7 (10  $\mu$ g/ml stock solution)** Reconstitute the contents of the vial to a concentration of 10  $\mu$ g/ml in sterile PBS containing 1% (wt/vol) BSA. Prepare aliquots and store them at  $-80^{\circ}\text{C}$  for up to 1 year.

**FGF18 (25  $\mu$ g/ml stock solution)** Reconstitute the contents of the vial to a concentration of 25  $\mu$ g/ml in sterile PBS containing 1% (wt/vol) BSA. Prepare aliquots and store them at  $-80^{\circ}\text{C}$  for up to 1 year.

**SHH (25  $\mu$ g/ml stock solution)** Reconstitute the contents of the vial to a concentration of 25  $\mu$ g/ml in sterile distilled water. Prepare aliquots and store them at  $-80^{\circ}\text{C}$  for up to 1 year.

**WNT3a (200  $\mu$ g/ml stock solution)** Reconstitute the contents of the vial to a concentration of 200  $\mu$ g/ml in sterile PBS containing 1% (wt/vol) BSA. Prepare aliquots and store them at  $-80^{\circ}\text{C}$  for up to 1 year.

**mTESR1** Prepare mTESR medium by mixing 100 ml of mTESR1 supplement with 400 ml of mTESR1 basal medium. **vCRITICAL** Make sure that the basal medium and supplement end with the same letter (i.e., same lot no.). The medium can be stored at  $4^{\circ}\text{C}$  for up to 2 weeks, or divide the solution into aliquots and store them at  $-20^{\circ}\text{C}$  for up to 6 months.

**Amiloride (30 mg/ml stock solution)** Dissolve 30 mg of amiloride powder in 1 ml of DMSO. Store the solution in aliquots at  $-20^{\circ}\text{C}$  for up to 1 year.

**Forskolin (4.1 mg/ml stock solution)** Dissolve 4.1 mg of forskolin powder in 1 ml of DMSO. Store the solution in aliquots at  $-20^{\circ}\text{C}$  for up to 1 year.

**IBMX (22.2 mg/ml stock solution)** Dissolve 22.2 mg of IBMX powder in 1 ml of DMSO. Store the solution in aliquots at  $-20^{\circ}\text{C}$  for up to 1 year.

**Genistein (13.5 mg/ml stock solution)** Dissolve 13.5 mg of genistein powder in 1 ml of DMSO. Store the solution in aliquots at  $-20^{\circ}\text{C}$  for up to 1 year.

**Iodide solution** Dissolve 20.4 g of sodium iodide, 4.8 g of HEPES, 2.0 g of glucose, 0.5 g of calcium nitrate and 0.3 g of potassium nitrate in 1 liter of double deionized water. Adjust the pH to 7.2 with sodium hydroxide.

**▲ CRITICAL** Make sure that the osmolarity of the solution is 300 mOsm. Filter the solution and store it at  $4^{\circ}\text{C}$  for up to 1 year.

**Nitrate solution** Dissolve 11.6 g of sodium nitrate, 4.8 g of HEPES, 2.0 g of glucose, 0.5 g of calcium nitrate and 0.3 g of potassium nitrate in 1 liter of double deionized water. Adjust the pH to 7.2 with sodium hydroxide.

**▲ CRITICAL** Make sure that the osmolarity of the solution is 300 mOsm. Filter the solution and store it at  $4^{\circ}\text{C}$  for up to 1 year. **▲ CRITICAL** Before use, add 100  $\mu$ l of stock amiloride solution to 100 ml of nitrate solution.

**cAMP agonist solution** Dissolve 10  $\mu$ l of forskolin stock solution, 10  $\mu$ l of IBMX stock solution and 10  $\mu$ l of genistein stock solution in 10 ml of nitrate solution. Freshly prepare and keep it for no more than 1 h at room temperature.

**Lysis buffer** Dissolve 4.0 g of Tris chloride, 4.4 g of sodium chloride and 0.15 g of EDTA in 500 ml of double-deionized water. Adjust the pH to 7.4 and store it at room temperature for up to 1 year. **▲ CRITICAL** Before lysis of cells, add 50  $\mu$ l of protease K inhibitor, 100  $\mu$ l of 10% (wt/vol) SDS and 50  $\mu$ l of Triton X-100 to 4.8 ml of lysis buffer.

**Laemmli buffer (5 $\times$  stock solution)** Dissolve 1.9 g of Trizma, 5.0 g of SDS, 1.0 g of DTT and a few drops of bromophenol blue in 25 ml of glycerol; adjust the final volume to 50 ml with double deionized water. Store the buffer at  $4^{\circ}\text{C}$  for up to 6 months.

**Running buffer** Add 100 ml of Tris-glycine-SDS buffer to 900 ml of double deionized water. Store the buffer at  $4^{\circ}\text{C}$  for up to 6 months.

**Transfer buffer** Dissolve 14.4 g of glycine and 3.0 g of Tris base in 800 ml of double deionized water. Add 200 ml of methanol to the solution. **▲ CRITICAL** Add methanol just before use; do not store this solution for the long term.

**PBS (10 $\times$  stock solution)** Dissolve 14.2 g of sodium phosphate dibasic and 87.7 g of sodium chloride in 1 liter of double deionized water. Adjust the pH to 7.2 and store it at room temperature for 1 year.

**PBST** Add 100 ml of PBS (10 $\times$ ) to 900 ml of double deionized water. Add 1 ml of Tween 20 and store it at room temperature for 1 year.

**Blocking buffer, 5% (wt/vol)** Dissolve 5 g of skim milk in 100 ml of PBST. Store the buffer at  $4^{\circ}\text{C}$  for 1 week.

**hESC/iPSC growth medium** Prepare ~500 ml of the growth medium. The medium should be used within 2 weeks; however, supplementation with additional FGF2 can be performed after 2 weeks (maximum storage is 4 weeks).

Composition	Volume	Final concentration
KnockOut DMEM	400 ml	
KnockOut serum replacement	100 ml	20%
Penicillin-streptomycin	3 ml	1%
Glutamax	6 ml	2 mM
Non-essential amino acid	3 ml	1 mM
MTG	15 $\mu$ l	0.15 mM
FGF2	100 $\mu$ l	20 ng/ml

**Differentiation basal medium** Prepare ~500 ml of the differentiation basal medium. Growth factors are supplemented to this medium for the stepwise differentiation process. Store the medium at  $4^{\circ}\text{C}$  for up to 2 months.

Composition	Volume	Final concentration
KnockOut DMEM	450 ml	
KnockOut serum replacement	50 ml	10%
Penicillin-streptomycin	3 ml	1%
Glutamax	6 ml	2 mM
MTG	15 $\mu$ l	0.15 mM
Non-essential amino acid	3 ml	1 mM

**BEGM** Prepare the medium according to the manufacturer's recommendation (from BEGM bullet kit). Thaw the frozen supplements provided with the medium at  $4^{\circ}\text{C}$  overnight before preparing the medium. Store it at  $4^{\circ}\text{C}$  for up to 1 month.

Composition	Volume (ml)
BEBM basal medium	500
BPE	2
Insulin	0.5
Hydrocortisone	0.5
GA-1000	0.5
Transferrin	0.5
Tri-iodothyronine	0.5
Epinephrine	0.5
Retinoic acid	0.5
Human epidermal growth factor (hEGF)	0.5

**B-ALI medium** Prepare the medium according to the manufacturer's recommendation (from B-ALI bullet kit). Thaw the frozen supplements supplied with the medium at 4 °C overnight before preparing the medium. Store it at 4 °C for up to 1 month.

Composition	B-ALI differentiation medium (ml)	B-ALI growth medium (ml)
Basal medium	500	250
Bovine pituitary extract (BPE)	2	1
Insulin	0.5	0.25
Hydrocortisone	0.5	0.25
GA-1000	0.5	0.25
Retinoic acid	0.5	0.25
Transferrin	0.5	0.25
Tri-iodothyronine	0.5	0.25
Epinephrine	0.5	0.25
hEGF	0.5	0.25

**Primers for real-time qRT-PCR characterization**

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
<i>KRT5</i>	GGAGTTGGACCAGTCAACATC	TGGAGTAGTAGCTTCCACTGC
<i>MUC5AC</i>	CCATTGCTATTATGCCCTGTGT	TGGTGGACGGACAGTCACT
<i>NKX2-1</i>	ACCAGGACACCATGAGGAAC	CGCCGACAGGTACTTCTGTT
<i>FOXJ1</i>	GAGCGGCGCTTTCAAGAAG	GGCCTCGGTATTCA CCGTC
<i>SFTPC</i>	CACCTGAAACGCCTTCTTATCG	TGGCTCATGTGGAGACCCAT
<i>CCSP</i>	TTCAGCGTGTCATCGAAACCCC	ACAGTGAGCTTTGGGCTATTTTT
<i>FOXA2</i>	AGGAGGAAAACGGGAAAGAA	CAACAACAGCAATG GAGGAG
<i>BACT</i>	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTACAATGCA
<i>SOX17</i>	AAGGGCGAGTCCCCTATC	TTGTAGTTGGGGTG GTCCTG
<i>TG</i>	AGAAGAGCCTGTGCTGAAA	TTGGACCAGAAGGAGCAGTC
<i>NKX6-1</i>	ATTCGTTGGGGATGACAGAG	CGAGTCTGCTCTCTCTGG
<i>PDX1</i>	CCCATGGATGAAGTCTACC	GTCCTCTCTCTTTT TCCAC
<i>AFP</i>	TGGGACCCGAACCTTTCCA	GGCCACATCCAGGACTAGTTTC
<i>CFTR</i>	CTATGACCCGGATAACAAGGAGG	CAAAAATGGCTGGGTGTAGGA
<i>NANOG</i>	TGATTTGTGGGCCTGAAGAAA	GAGGCATCTCAGCA GAAGACA
<i>TP63</i>	ACTTCACGGTGTGCCACCCT	GAGCTGGGGTTTCTACGAAACGCT

<i>CDX2</i>	CTGGAGCTGGAGAAGGAGTTTC	ATTTTAACCTGCCTCTCAGAGAGC
<i>SOX2</i>	GCACATGAAGGAGCACCCGGA TTA	CGGGCAGCGTGTACTTATCCTTCTT
<i>FOXG1</i>	CTCCGTCAACCTGCTCGCGGF	CTGGCGCTCATGGA CGTGCT
<i>PAX9</i>	TGGTTATGTTGCTGGACATGG GTG	GGAAGCCGTGACAG AATGACTACCT
<i>SOX9</i>	GAGGAAGTCGGTGAAGAACG	CCAACATCGAGACC TTCGAT
<i>PAX6</i>	TCTTTGCTTGGGAAATCCG	CTGCCCCGTTCAACA TCCTTAG
<i>OTX2</i>	GTGGGCTACCCGGCCACCC	GCACCTCGACTCG GGCAAG
<i>KRT15</i>	GGCTGGAGAACTCACTGGC	CAGGCTGCGGTAAG TAGCG
<i>KRT16</i>	GACCGGCGGAGATGTGAAC	CTGCTCGTACTGGT CACGC
<i>MUC16</i>	CCAGTCTACATCTTCGGTTGT	AGGGTAGTTCCTAG AGGGAGTT
<i>PDPN</i>	GTCCACGCGCAAGAACAAAG	GGTCACTGTTGACA AACCATCT
<i>P2X7</i>	TATGAGACGAAACAAAGTCACTCG	GCAAAGCAAACGTA GGAAAAGAT
<i>FOXA1</i>	CTCGCCTTACGGCTCTACG	TACACACCTTGGTA GTACGCC
<i>FOXP2</i>	AATCTGCGACAGAGACAATAAGC	TCCACTTGTTTGTCTGTAAA
<i>FOXE1</i>	CACGGTGGACTTCTACGGG	GGACACGAACCGAT CTATCCC
<i>PITX1</i>	CTAGAGGCCACGTTCCAGAG	TGGTTACGCTCGCG CTTAC
<i>DLX3</i>	CTCGCCCAAGTCGGAATATAC	CTGGTAGCTGGAGT AGATCGT
<i>MUC2</i>	AGGATGACACCATCTACCTCAC	CATCGCTCTTCTCA ATGAGCA
<i>TBX1</i>	CGGCTCCTACGACTATTGCC	GGAACGTATTCTTCTT GCTTGCCCT

**Positive control human RNA used for real-time qRT-PCR**

Tissue name	Tissue type	Biochain catalog no.
Lung	Fetal	R1244152-50
Thyroid	Fetal	R1244265-10
Esophagus	Adult	R1234106-60
Pancreas	Adult	R1234188-50
Liver	Fetal	R1244149-50
Trachea	Adult	R1234160-50
Skin	Adult	R1234218-50
Brain	Fetal	R1244035-50

## PROTOCOL

### EQUIPMENT SETUP

**Gelatin-coated plate** Add 2 ml of 0.1% (wt/vol) gelatin solution to each well of the six-well culture plate. Ensure that the gelatin coats the entire surface area of the wells, and leave the plate at 37 °C for 1 h before use. Remove excess gelatin before use.

**Matrigel-coated Petri dish** Transfer 20 ml of cold DMEM/F12 to a 50-ml tube on ice. Thaw a 250- $\mu$ l aliquot of Matrigel on ice; once it is thawed, cool a pipette tip by pipetting cold DMEM/F12 up and down several times, and then transfer the Matrigel to the DMEM/F12 on ice. Cap the 50-ml tube and mix well by inverting the tube five or six times. By using a cooled pipette, transfer 3 ml of Matrigel to the 10-cm Petri dish or 1 ml of Matrigel per well of the six-well-plate and rock the dish to disperse the Matrigel evenly across the surface. Leave it at room temperature for 2 h to coat. Seal the dish with Parafilm and store it at 4 °C for up to 1 week. Warm the dish for 1 h at room temperature before use.

**Collagen-coated Transwells** Thaw 1 ml of collagen stock and dilute 1:10 to a working stock concentration of 60  $\mu$ g/ml with deionized water. The working collagen stock can be stored at 4 °C for up to 4 weeks. Add 150–200  $\mu$ l of collagen per Transwell directly onto the membrane, by making sure that the membrane is evenly covered with collagen solution, and leave it at room temperature for a minimum of 18 h. Collagen-coated Transwells can be stored in a sterile environment at room temperature for up to 3 d. Before use, remove excess collagen from the surface and wash the membrane three times with DMEM/F12, and allow it to air-dry for 1 h at room temperature. **▲ CRITICAL** It is important to wash the Transwells carefully before use, as the remaining collagen solution is acidic; it can reduce the efficiency of cell attachment and cause cell death.

**▲ CRITICAL** It is best to prescreen a lot before buying a large quantity. Lots can vary in terms of potency of cell attachment and possible toxicity to cells.

**▲ CRITICAL** Too much collagen solution can reduce polymerization of the collagen, and it can result in inadequate coating of the membrane.

### PROCEDURE

#### Adaptation of hESCs or hiPSCs to single-cell passaging ● TIMING 2 h

- 1| Before this step, cells should be maintained under normal PSC conditions on MEFs in hESC/iPSC growth medium.
- 2| To adapt cells to single-cell culture conditions, wash confluent cells with PBS  $-Ca^{2+}-Mg^{2+}$  twice. Add 1 ml of TrypLE to each well of a six-well culture plate, and then incubate the plate at 37 °C for 5 min. The borders of the colonies should look rounded, like the colonies are about to peel off but are not yet detached from the plate.  
**▲ CRITICAL STEP** Optimal treatment time with TrypLE must be determined for individual cell lines to yield viable single cells that can be expanded in culture.  
**? TROUBLESHOOTING**
- 3| Carefully remove TrypLE and add 5 ml of growth medium. By using a P1000 pipette, gently dissociate the colonies from the plate by triturating gently up and down about ten times to achieve a single-cell suspension.  
**? TROUBLESHOOTING**
- 4| Transfer the cells to a 15-ml tube and centrifuge at 200g for 5 min at room temperature to obtain a cell pellet.
- 5| Resuspend the cell pellet in hESC/iPSC growth medium by gentle trituration, and passage it at a 1:6 split ratio to a new six-well plate containing MEFs.  
**? TROUBLESHOOTING**
- 6| Continue to passage the cells as described above (Steps 1–5) by using TrypLE to obtain a single-cell suspension. Cells should be passaged weekly at a 1:6 split ratio and maintained in hESC/iPSC growth medium on MEFs until stable cultures can be achieved.  
**▲ CRITICAL STEP** It is essential to have cells fully adapted to single-cell culture conditions before proceeding. Cells will be viable when plated as single cells onto feeders.  
**? TROUBLESHOOTING**

#### Passaging of hESCs or hiPSCs to Matrigel ● TIMING 2 h

- 7| Precoat Matrigel plates when hESCs or hiPSCs are ready for differentiation. Prepare 10-cm dishes coated with Matrigel overnight at 4 °C. Follow the instructions in Equipment Setup carefully.  
**▲ CRITICAL STEP** Handle the Matrigel on ice, as the gel will solidify at room temperature.
- 8| Before passaging the cells, bring the Matrigel plate to room temperature for at least 1 h.
- 9| Passage the cells by washing them twice with PBS  $-Ca^{2+}-Mg^{2+}$  and add 1 ml of TrypLE to each well of a six-well culture plate. Incubate it at 37 °C for 5 min or at treatment time as determined previously for individual lines.
- 10| Carefully remove TrypLE and add 5 ml of growth medium. By using a P1000 pipette, gently dissociate the colonies from the plate by triturating up and down to obtain single cells.

11| Centrifuge the mixture at 200g for 5 min at room temperature to pellet the cells, and then resuspend the cell pellet in mTESR1 medium by gentle trituration. Passage one well of a six-well plate onto a 1 × 10 cm dish precoated with Matrigel in mTESR1 medium.

? TROUBLESHOOTING

12| Grow the cells in mTESR1 until they are confluent, and passage them at a 1:3 ratio onto a fresh 10-cm plate, as described in Steps 7–11, for an additional two passages.

▲ **CRITICAL STEP** This is to reduce the amount of MEF cell transfer to the differentiation cultures; excess MEFs can inhibit proper differentiation.

Passaging cells for differentiation ● **TIMING 30 min**

13| *Day -1.* Freshly prepare a 10-cm Matrigel plate by coating it with Matrigel overnight at 4 °C.

14| *Day 0.* Before passaging cells, bring the Matrigel plate to room temperature for at least 1 h.

15| Wash the hESCs or hiPSCs with PBS –Ca<sup>2+</sup>–Mg<sup>2+</sup> 2×. Add 3 ml of TrypLE and incubate at 37 °C for 5 min.

16| Pipette the cells up and down five times using a P1000 tip to ensure that cells are detached from the plate and that they are in a single-cell suspension.

17| Transfer the cells to a 15-ml conical tube and add an equal volume of warmed DMEM/F12. Centrifuge the tube at 200g for 5 min at room temperature.

18| Aspirate the supernatant and resuspend the cell pellet in 1 ml of DMEM/F12. Take 10 μl for cell count using the hemocytometer.

19| Plate cells onto a precoated 10-cm dish at a density of 2.5 × 10<sup>5</sup> per cm<sup>2</sup> and incubate in the cell culture incubator at 37 °C overnight.

▲ **CRITICAL STEP** The cells should be >90% confluent the next day.

? TROUBLESHOOTING

Stage 1, differentiation to DE ● **TIMING 5 d**

20| *Day 1, morning.* Prepare medium 1 by adding 100 μl of supplement A and 100 μl of supplement B to 9.8 ml of StemDiff medium (as per the manufacturer’s protocol).

▲ **CRITICAL STEP** Supplements should be thawed and kept on ice. Store at 4 °C until use.

21| Aspirate the medium from the dish and wash the cells once with warmed DMEM/F12.

22| Add 10 ml of medium 1 per 10-cm dish of cells, and incubate in the cell culture incubator at 37 °C overnight.

23| *Day 2, morning.* Prepare medium 2 by adding 100 μl of supplement B to 9.9 ml of differentiation basal medium per 10-cm dish.

▲ **CRITICAL STEP** Medium 2 will be used for the next 3 d, but it should be freshly made every day.

24| Aspirate medium 1 from the cells and add 10 ml of medium 2 to the cells. Incubate the medium in the cell culture incubator at 37 °C overnight.

25| *Day 3, morning.* Repeat Steps 23 and 24.

26| *Day 4, morning.* Repeat Steps 23 and 24.

27| *Day 5.* Assess morphological changes and assay for DE markers by FACS and/or RT-PCR (**Box 1**). Morphology of cells should be as shown in **Figure 2**.

28| Prepare collagen-coated Transwells and leave them in the biosafety cabinet overnight to coat. Follow the instructions in Equipment Setup carefully.

? TROUBLESHOOTING



## PROTOCOL

### Stage 2, differentiation to the anterior foregut endoderm ● TIMING 10 d

29| Prepare anterior foregut endoderm (AFE) medium by adding FGF2 (500 ng/ml) and SHH (50 ng/ml) to prewarmed differentiation basal medium.

▲ **CRITICAL STEP** AFE medium must be freshly made with every use.

30| *Day 6, morning.* Aspirate medium 2 from the cells and wash with PBS ( $-Ca^{2+}$ – $Mg^{2+}$ ) twice.

31| Add 2 ml of TrypLE and incubate the cells at 37 °C for <5 min (until the cells start to detach).

? **TROUBLESHOOTING**

32| Add 8 ml of basal differentiation medium and transfer the cells to a 15-ml conical tube. Centrifuge the cells at 400g for 5 min at room temperature.

33| Aspirate the supernatant and resuspend in 1 ml of AFE medium.

34| Pipette 10  $\mu$ l and load it onto the hemocytometer to count the cells. Dilute the cell suspension with AFE medium to 2,000 cells per  $\mu$ l.

▲ **CRITICAL STEP** Be very gentle when triturating the cells, as excess cell death can occur.

? **TROUBLESHOOTING**

35| Gently transfer 250  $\mu$ l of the cell suspension ( $\sim 5 \times 10^5$  cells) to the apical side of the collagen-coated Transwells. Ensure that collagen-coated wells are washed with PBS before use to remove residual collagen solution. A 10-cm dish of confluent DE cells can generate two plates of 12-well Transwell cultures.

▲ **CRITICAL STEP** Make sure that the cell volume coats the entire membrane.

36| Incubate the cells in the cell culture incubator at 37 °C.

37| *Day 7, morning.* Check the cells under the microscope. Change the medium on cells by removing the AFE medium from the apical side of the Transwell and replacing it with 0.5 ml of fresh AFE medium. Only add medium to the apical side of the Transwell for subsequent medium changes (Steps 38–49).

▲ **CRITICAL STEP** Adding medium to the basolateral side may reduce cell attachment to the membrane.

▲ **CRITICAL STEP** On this day, the cells should be attached to the membrane. Unfortunately, many technically sensitive problems may arise that prevent or limit cell attachment to the membrane.

? **TROUBLESHOOTING**

38| *Day 8, morning.* Carefully aspirate old AFE medium from the apical side of the cells and add 0.5 ml of freshly made AFE medium per Transwell. Incubate in the cell culture incubator at 37 °C for 48 h.

? **TROUBLESHOOTING**

39| *Day 10, morning.* Carefully aspirate old AFE medium from the cells and add 0.5 ml of freshly made AFE medium per Transwell. Incubate the medium in the cell culture incubator at 37 °C for 24 h.

40| *Day 11, morning.* Take a subset of the cultures and assay for AFE markers by immunofluorescence or RT-PCR (**Box 1**). Results obtained should be as shown in **Figure 3**. Proceed to the next step with parallel cultures while the characterization is performed on a subset of the cultures.

### Stage 3a, differentiation to lung progenitors ● TIMING 5 d

41| Prepare stage 3a medium by adding FGF7 (50 ng/ml), FGF10 (50 ng/ml) and BMP4 (5 ng/ml) to differentiation basal medium.

▲ **CRITICAL STEP** Medium must be made freshly with every use. Note that high levels of FGF7 appear to induce a mesenchyme phenotype that will take over the culture.

42| *Day 11, afternoon.* Aspirate AFE medium from the cells and add freshly made stage 3a medium (0.5 ml per Transwell to the apical side). Incubate the cells in the cell culture incubator at 37 °C for 48 h.

▲ **CRITICAL STEP** When changing the medium, be very careful to not aspirate the layer of cells on the membrane. In addition, remove any medium that has leaked to the basal side of the Transwell.

## Box 1 | Characterization of differentiated cells at each stage

1. After each stage, collect a set of cells for characterization by flow cytometry (step 1A) and/or immunofluorescence staining (step 1B) and/or real-time qRT-PCR (step 1C). At the final stage of differentiation, test for CFTR function by iodide efflux measurement (step 1D) or assay for the presence of mature CFTR protein by western blotting (step 1E). Note that cells that have been tested for function can be subsequently lysed for western blotting.

**▲ CRITICAL STEP** Ensure that sufficient cultures are set up at the start of stage 1 to allow for characterization at the end of each stage.

### (A) Flow cytometric analysis ● TIMING 3 h

(i) Aspirate the differentiation medium and wash the cells twice with PBS ( $-Ca^{2+}-Mg^{2+}$ ).

(ii) Remove PBS and add 0.5–2 ml of TrypLE to each culture (depending on culture plate size). Incubate the cells with TrypLE for 5 min at 37 °C.

(iii) Stop the enzymatic activity by adding 5× (vol/vol) FACS buffer.

(iv) Transfer the cells to a 15-ml conical tube and centrifuge the cells at 400g for 5 min at room temperature.

(v) Wash the cells once with 3 ml of FACS buffer, and then transfer them to an FACS tube containing a filter top to remove cell clumps and to ensure a single-cell suspension.

(vi) Centrifuge the cells at 400g for 5 min at room temperature.

(vii) Resuspend the cell pellet in 1 ml of FACS buffer.

(viii) Pipette 10 µl of the cell suspension and count the cell number using a hemocytometer.

(ix) Divide the cell suspension into aliquots in FACS tubes at a concentration of up to  $1 \times 10^6$  cells per 100 µl into each tube.

(x) Incubate the aliquots of cell suspension with primary antibodies in the dark at 4 °C for 30 min. Use the antibody concentrations listed in the Reagents section.

**▲ CRITICAL STEP** Include appropriate nonstained, isotype and single-antibody compensation controls (if multiplex staining is performed). For multiplex staining, consult an expert FACS operator to set up the most ideal multicolor fluorescence combination that would minimize compensation needs. To avoid false positive results, use antibodies that are directly conjugated to a fluorophore (if possible).

(xi) At the end of the primary antibody stain, add 500 µl of FACS buffer and centrifuge the cells at 400g for 5 min at room temperature.

(xii) Wash the cells again with 500 µl of FACS buffer, and centrifuge the cells at 400g for 5 min at room temperature.

(xiii) If primary antibodies are not directly conjugated to a fluorophore, perform secondary antibody staining using the concentrations listed in Reagents section at 4 °C in the dark for 30 min in a 100-µl volume.

(xiv) At the end of the secondary antibody staining, add 500 µl of FACS buffer and centrifuge the cells at 400g for 5 min at room temperature.

(xv) Wash the cells again with 500 µl of FACS buffer, and centrifuge the cells at 400g for 5 min at room temperature.

(xvi) Resuspend the cells in 500 µl of 1% (vol/vol) PFA.

**■ PAUSE POINT** Flow cytometric analysis can be performed immediately, or the cells can be analyzed later (up to 24 h). Store the cells at 4 °C in the dark.

(xvii) Perform flow cytometric analysis. We used the BD Biosciences LSRII analyzer with the following laser setup: Blue/488 nm, A488/FITC 505LP-515/20BP, PE 550LP-575/25BP; Green/532 nm, PE-Texas Red/PI 600LP-610/20BP, PE-Gr 575/26BP; Red/639 nm, APC/A633 660/20BP. Data analysis was performed using FlowJo software.

### (B) Immunofluorescence staining ● TIMING 2 d

(i) Remove the medium from the membrane and wash the cells once with PBS ( $-Ca^{2+}-Mg^{2+}$ ).

(ii) Fix the membrane containing the cells with 4% (vol/vol) PFA for 10 min at room temperature. Add 500 µl of PFA on the apical side of the Transwell and 1 ml of PFA to the basolateral side of the Transwell. After incubation, remove PFA and wash twice with PBS for 3 min per wash.

**▲ CRITICAL STEP** Make sure that PFA has equilibrated to room temperature before adding to the cells. For CFTR staining, use ice-cold methanol (100%) to fix instead of PFA, fix for 10 min in  $-20$  °C, wash twice with PBS for 3 min per wash.

**■ PAUSE POINT** The cells can be stored in PBS for up to 1 week at 4 °C.

(iii) Permeabilize cells with 0.25% (vol/vol) Triton X-100 solution (diluted in PBS) by adding 200 µl of solution to each well of cells and incubating them at room temperature for 15 min.

**▲ CRITICAL STEP** This step only applies to PFA-fixed cells, and not to methanol-treated cells, as methanol also permeabilizes the cells.

(iv) Block with 200 µl of blocking buffer containing 5% (vol/vol) normal goat serum in 0.25% (vol/vol) Triton X-100 solution for 30 min at room temperature.

(v) Add primary antibodies at the concentrations listed in Reagents section to the wells. Incubate the cells in primary antibody overnight at 4 °C.

**▲ CRITICAL STEP** Include appropriate isotype controls for each primary antibody.

(vi) Wash the cells twice with PBS for 3 min at room temperature.

(vii) Add secondary antibodies in 200 µl of PBS and incubate at room temperature for 1 h.

(viii) Wash the cells twice with PBS for 3 min at room temperature.

(ix) Counterstain with nuclear stain DAPI (1:1000 in PBS) for 10 min covered at room temperature.

(x) Wash the cells once with PBS for 3 min at room temperature.

(continued)

**Box 1 | (continued)**

(xi) Cut the membrane out of the Transwell insert using a scalpel blade and cut the edge of the membrane, being careful not to rip it.

**! CAUTION** Be careful while working with the scalpel to remove the membrane.

(xii) Mount the membrane onto a glass slide by placing cell-side up onto 100 µl (~2 drops) of immunofluorescence mounting medium.

(xiii) Add another 100 µl of immunofluorescence mounting medium on top of the membrane and cover with a glass coverslip. Be sure not to introduce air pockets between the membrane and the glass coverslip.

**■ PAUSE POINT** Capture images of the cells within the next 3 d to avoid degradation and loss of signal of the fluorophore over time. Store the slides at 4 °C until imaging.

(xiv) Image the cells with a confocal microscope using the Velocity software.

**(C) Real-time qRT-PCR assessment ● TIMING 2 d**

(i) Collect cells by washing the cells twice with PBS (–Ca<sup>2+</sup>–Mg<sup>2+</sup>).

(ii) Remove the PBS and add 0.5–2 ml of TrypLE to each culture (depending on culture plate size). Incubate the cells with TrypLE for 5 min at 37 °C.

(iii) Stop the enzymatic activity by the addition of culture medium.

(iv) Transfer the cells to a 15-ml conical tube and centrifuge the cells at 400g for 5 min at room temperature.

(v) Remove the supernatant and resuspend the cells in 350 µl of RLT lysis buffer (in the RNeasy kit). Mix the lysed cells thoroughly by pipetting up and down five times.

**■ PAUSE POINT** Lysed cells can be stored at –20 °C for up to 6 months.

(vi) To ensure that cells are lysed and that DNA/RNA is released into the solution, pipette the solution up and down an additional ten times.

(vii) Add equal volumes of 70% (vol/vol) ethanol and follow the manufacturer’s protocol for RNA isolation.

(viii) Once RNA has been eluted, determine the RNA concentration using a NanoDrop 2000c.

**▲ CRITICAL STEP** The ratio of absorbance 260/280 should be 2.0 for RNA.

(ix) To generate cDNA from RNA, calculate the volume of RNA for 1 µg per sample of reverse transcription using Superscript II enzyme. To a thin-walled PCR tube, add the following:

Component	Amount per sample	Final
RNA	X µl	1 µg
Oligo(dT) <sub>12–18</sub>	1 µl	500 µg/ml
dNTP mix	1 µl	10 mM each
Sterile ddH <sub>2</sub> O	Up to 12 µl	
Total volume	13 µl	

(x) Heat the mixture to 65 °C for 5 min and quickly chill it on ice.

(xi) Add the following mixture to the tube of chilled RNA.

Component	Amount per sample	Final
5× First-strand buffer	4 µl	1×
DTT	2 µl	0.1 M
Superscript II	1 µl	200 units

(xii) Mix gently.

**▲ CRITICAL STEP** Be sure to include positive control RNA in this reverse-transcription (RT) reaction. This will serve as your control for gene expression in the PCR.

(xiii) Transfer PCR tubes to Thermal Cycler and perform the reverse-transcription reaction as follows:

Cycle number	Temperature (°C)	Duration
1	42	50 min
2	70	15 min
3	4	Forever

(xiv) Pipette 1 µl of cDNA and determine the concentration using a NanoDrop 2000c.

(xv) Dilute cDNA to 50 ng/µl and keep it on ice until it is ready to use.

(xvi) Prepare a map of the PCR plate and calculate the total number of samples including no-template control, no-RT control and positive controls in your calculation for each primer pair reaction (gene). Run duplicate or triplicate reactions per sample.

(xvii) Prepare a real-time PCR master mix as follows:

**▲ CRITICAL STEP** Always prepare extra master mix, as it often sticks to the tube/pipettes. Formulation per sample is as follows:

Component	Amount per sample	Final
Roche 2× SYBR Green mix	6 µl	1×
Forward and reverse primer mix	0.6 µl	20 µM each
RNA/DNase-free distilled water	0.4 µl	
Total volume	7 µl	

(continued)



## Box 1 | (continued)

- (xviii) Mix and add 7  $\mu$ l of master mix to each well of a 96-well PCR plate.
- (xix) Add 5  $\mu$ l of cDNA (50 ng/ $\mu$ l) to the allocated wells as per your map.
- (xx) Cover the plate with sealing tape and centrifuging briefly at 1,000 r.p.m. for 1 min.
- (xxi) Transfer the plate to the LightCycler 480.

Program name	Cycle(s)	Temperature ( $^{\circ}$ C)	Duration (00:00, min:s)	Ramp rate ( $^{\circ}$ C/s)
Preincubation	1	95	05:00	4.4
Amplification	45	95	00:10	4.4
		60	00:05	2.2
		72	00:05	4.4
Melt curve	1	95	00:05	4.4
		65	01:00	2.2
		95	Continuous	0.11
Cooling	1	40	00:10	1.5
END				

(xxii) At the end of the PCR, check the dissociation curve for each sample to ensure that there are no primer dimers (seen in the no-template control) and no amplification in no-RT controls. Dissociation curve should show one single peak at the appropriate melting temperature.

### (D) Iodide efflux measurement for CFTR activity ● TIMING 3 h

- (i) Wash apical and basolateral surfaces of cultures with 4 ml of 1 $\times$  PBS ( $-Ca^{2+}$ - $Mg^{2+}$ ) twice.
- (ii) Load the basolateral side of cultures with 850  $\mu$ l of iodide solution for 1 h at 37  $^{\circ}$ C.
- (iii) After 1 h of incubation, wash the apical and basolateral surfaces with 4 ml of nitrate solution 10 times.
- (iv) Start the discontinuous iodide efflux experiment time course as follows:  
Part 1: add 350  $\mu$ l of nitrate solution and collect the solution into individual wells of a 96-well plate at each minute for 3 min.  
Part 2: add 350  $\mu$ l of cAMP agonist solution and collect the solution into individual wells of a 96-well plate at each minute for 5 min.
- (v) Read the iodide concentration of the collected sample solutions using the iodide-sensitive combination microelectrode and the Digidata 1320A data acquisition system with the Clampex 8.1 program.

▲ **CRITICAL STEP** Wash the iodide-sensitive combination microelectrode with double deionized water thoroughly between readings.

▲ **CRITICAL STEP** Make a standard curve for iodide concentration to convert readout to iodide concentration.

### ? TROUBLESHOOTING

### (E) Western blotting for CFTR protein ● TIMING 2 d

- (i) Remove the medium from the membrane and wash the cells once with PBS ( $-Ca^{2+}$ - $Mg^{2+}$ ).
- ▲ **CRITICAL STEP** If you are using samples that were previously used for iodide efflux assay, wash the apical and basolateral surfaces of the cultures with 4 ml of 1 $\times$  PBS ( $-Ca^{2+}$ - $Mg^{2+}$ ) two times.
- (ii) Add 200  $\mu$ l of lysis buffer at the apical surface of each culture. Shake at 4  $^{\circ}$ C for 20 min.
- (iii) Pipette the solution up and down and transfer the supernatant to microcentrifuge tubes.
- (iv) Spin down the insoluble fraction at 10,000g for 10 min at room temperature.
- (v) Remove 40  $\mu$ l of the supernatant and add 10  $\mu$ l of Laemmli buffer to the supernatant.
- **PAUSE POINT** Store the resulting supernatant at  $-20^{\circ}$  C for up to 1 year.
- (vi) Run the samples on a 6% (wt/vol) SDS-PAGE gel in running buffer.
- (vii) Transfer the proteins on a nitrocellulose membrane in transfer buffer for 1 h at 100 V.
- (viii) Block the membrane with 10 ml of blocking solution for at least 1 h at room temperature, with shaking.
- (ix) Incubate the membrane with CFTR antibody no. 596 at the concentrations listed in Reagents section diluted in blocking solution. Incubate overnight at 4  $^{\circ}$ C with gentle shaking.
- (x) Wash the membrane three times with PBST for 10 min at room temperature, with shaking.
- (xi) Incubate the membrane with secondary goat anti-mouse Ig HRP for at least 1 h at room temperature with shaking.
- (xii) Wash the membrane with PBST for 10 min at room temperature three times.
- (xiii) Expose the blot with enhanced chemiluminescence on the film.

### ? TROUBLESHOOTING

**43| Day 13, morning.** Aspirate stage 3a medium from cells and add freshly made stage 3a medium (0.5 ml per Transwell). Incubate the cells in the cell culture incubator at 37  $^{\circ}$ C for 48 h.

**44| Day 15, morning.** Aspirate stage 3a medium from the cells and add freshly made stage 3a medium (0.5 ml per Transwell). Incubate the cells in the cell culture incubator at 37  $^{\circ}$ C for 24 h.

## PROTOCOL

### Stage 3b, differentiation to immature lung cells ● TIMING 5 d

45| Prepare stage 3b medium by adding FGF7 (10 ng/ml), FGF10 (10 ng/ml) and FGF18 (10 ng/ml) to the differentiation basal medium.

▲ **CRITICAL STEP** The medium must be made freshly with every use.

46| *Day 16, morning.* Aspirate old stage 3a medium from the cells and add fresh stage 3b medium (0.5 ml per Transwell to the apical side). Incubate the cells in the cell culture incubator at 37 °C for 48 h.

▲ **CRITICAL STEP** When changing the medium, be very careful to not aspirate the layer of cells on the membrane. In addition, remove any medium that has leaked to the basal side of the Transwell.

47| *Day 18, morning.* Aspirate stage 3b medium from the cells and add fresh stage 3b medium (0.5 ml per Transwell). Incubate the cells in the cell culture incubator at 37 °C for 48 h.

48| *Day 20, morning.* Aspirate old stage 3b medium from the cells and add fresh stage 3b medium (0.5 ml per Transwell). Incubate the cells in the cell culture incubator at 37 °C for 24 h.

49| *Day 21, morning.* Take a subset of the cells and assay for lung epithelial markers by immunofluorescence and/or RT-PCR (**Box 1**). Results obtained should be as shown in **Figure 5**. Proceed to the next step with parallel cultures while the characterization is performed on a subset of the cultures.

### Stage 4, expansion of immature lung cells ● TIMING 10 d

50| Prepare BEGM medium by thawing the supplements at 4 °C overnight. Add thawed supplements to the BEGM basal medium.

▲ **CRITICAL STEP** Only prewarm the required volume of complete BEGM before use. Excess warming of this medium could result in the degradation of growth components.

51| Add FGF18 (10 ng/ml) to the BEGM medium and warm it in a water bath before use. Freshly prepare the medium with every use.

52| *Day 21, afternoon.* Aspirate old stage 3b medium from cells and add freshly prepared BEGM + FGF18 (0.5 ml per Transwell). Incubate the cells in the cell culture incubator at 37 °C for 48 h.

▲ **CRITICAL STEP** When changing the medium, be very careful to not aspirate the layer of cells on the membrane. In addition, remove any medium that has leaked to the basal side of the Transwell. The medium should only be added to the apical side of the Transwells.

53| *Day 23, morning.* Aspirate old BEGM + FGF18 from cells and add freshly made medium (0.5 ml per Transwell). Incubate the cells in the cell culture incubator at 37 °C for 48 h.

54| *Day 25, afternoon.* Aspirate old BEGM + FGF18 from the cells and add freshly made medium (0.5 ml per Transwell). Incubate the cells in the cell culture incubator at 37 °C for 24 h.

55| *Day 26, morning.* Prepare B-ALI growth and differentiation medium according to the manufacturer's protocol. Aspirate old BEGM growth medium from the cells and add fresh B-ALI growth medium (0.5 ml to the apical side of the Transwell). Incubate the cells in the cell culture incubator at 37 °C for 48 h.

56| *Day 28, morning.* Aspirate old B-ALI growth medium from the cells and add fresh medium (0.5 ml per Transwell). Incubate the cells in the cell culture incubator at 37 °C for 48 h.

### ? TROUBLESHOOTING

57| *Day 30, morning.* Aspirate old B-ALI growth medium from the cells and add fresh medium (0.5 ml per Transwell). Incubate the cells in the cell culture incubator at 37 °C for 24 h.

58| *Day 30.* Assay for lung epithelial markers by immunofluorescence and/or RT-PCR on a subset of the cultures (**Box 1**). Results should be as described in **Figure 6**.

### Stage 5, maturation and polarization of the epithelium ● TIMING up to 5 weeks

59| Aspirate old B-ALI growth medium from the cells and add fresh B-ALI differentiation medium (1.5 ml per Transwell) only to the basolateral side of the Transwell. Incubate the cells in the cell culture incubator at 37 °C for 48 h.

**60** | *Day 31, morning.* Carefully remove B-ALI from the basolateral side of the Transwell, as well as any medium on the apical side of cells. Add fresh B-ALI differentiation medium (1.5 ml per Transwell) only to the basolateral side of the Transwell. Incubate the cells in the cell culture incubator at 37 °C for 48 h.

**▲ CRITICAL STEP** The epithelium peels from the membrane very easily, so take extra caution when changing the medium on these cells.

**? TROUBLESHOOTING**

**61** | Repeat Step 60 for up to 5 weeks. High expression of genes associated with *CFTR*-expressing ciliated epithelium should be detected after 5 weeks of ALI (**Fig. 7a**) provided that the epithelium does not rip off the membrane (**Box 1**).

**? TROUBLESHOOTING**

**? TROUBLESHOOTING**

Troubleshooting advice can be found in **Table 1**.

**TABLE 1** | Troubleshooting table.

Step	Problem	Possible reason	Solution
2	Detachment of colonies from MEFs	Excessive treatment with TrypLE	Ensure that the length of treatment does not exceed 10 min
3	hESCs/hiPSCs do not detach	Insufficient treatment with TrypLE	Increase the length of treatment to up to 10 min
5	Excessive cell death	This is normal when the cells are initially trying to adapt to trypsin-mediated passaging	Add ROCK inhibitor to improve the survival of cells after passaging
6	Inability to adapt cells to single-cell culture conditions	Line-to-line variability in the ability to adapt to trypsin-mediated passaging	Add ROCK inhibitor to improve survival of cells after passaging Optimize the timing of TrypLE incubation for each cell line
11	Insufficient cell number attachment to Matrigel-coated plate	Poor Matrigel coating of plate or too vigorous trituration of cells to achieve single-cell suspension	Optimize the Matrigel coating. To ensure single-cell suspension, briefly incubate the cell suspension with TrypLE again to break cell-to-cell adhesion
19, 34	Cultures do not achieve confluency	Inaccurate cell counting or cell death	Addition of live/dead stain during cell counting to ensure that plating density is based on viable cells Add ROCK inhibitor to improve the survival of cells after passaging
28	Ineffective collagen coating	It is important that not too much collagen solution is used to coat the well, as this reduces the ability of collagen to polymerize	Reduce the volume of collagen solution used to coat the well
31	DE cells do not detach, or there is too much cell death from enzyme treatment	Timing of TrypLE incubation may be too short or too long	Every minute, check to determine whether the cell borders appear to be detaching (but cells have not detached). At this point, continue with Step 32
37	Lots of floating cells and/or no cell attachment	Leftover acidic collagen solution on the membrane, which killed the cells	Ensure that the membrane is thoroughly washed before seeding cells Use medium with phenol red to test whether acidic collagen solution still remains, and perform extra washes as required
		Ineffective collagen coating	Test collagen coating before starting the protocol Order a bulk batch of the optimal collagen solution, as this may be a collagen batch issue

(continued)



**TABLE 1** | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
38	Lots of floating dead cells	This is normal, but too much cell death can also be toxic for live cells	Add ROCK inhibitor to improve survival of cells
56	Cells are confluent and appear to be growing on top of one another	Rapid cell growth	Change to ALI cultures to induce differentiation and maturation of the cells
60	Thick mucus layer on the apical side of the membrane, making it difficult to aspirate out the liquid	Too much mucus production, making the apical liquid viscous, which can peel the cells off the membrane if aspiration is done carefully	Instead of vacuum suction, use a manual pipette to carefully remove the apical liquid
61	Cells appear to be peeling off the membrane	This is common due to mucus production by the cells; as ALI culture continues this may worsen. It is hard to control cell growth as there is line-to-line variability	Determine the optimal cell density to seed the cells at Step 35
<b>Box 1,</b> step 1D(v)	Iodide baseline is too high or saturated	Inefficient washing of extraneous iodide	Increase the number of washes after iodide loading
<b>Box 1,</b> step 1E(xiii)	No CFTR signal on film	Very small amount of CFTR in samples	Increase the concentration of primary antibody no. 596 and secondary antibody goat anti-mouse monoclonal

● **TIMING**

See **Figure 1** for timing details for the PROCEDURE.

**ANTICIPATED RESULTS**

Careful monitoring of morphological and phenotypic changes in cultures while performing this protocol can greatly increase the chance of successful differentiation. Morphologically, cells convert from small high nuclear-to-cytoplasm content to a small nucleus and large cytoplasm cuboidal morphology shape as the cell transition from hESCs/hiPSCs to epithelial cells occurs. Evidence of morphological changes can be observed as early as day 3 during DE differentiation (**Fig. 2**). This method of monolayer differentiation should yield anywhere from 75 to >95% DE induction (CXCR4+CD117+; SOX17+FOXA2+) depending on the cell line. In our experience, the efficiency of DE generation generally is highest with hESC lines CA1>HES2>H9>CA2 compared with our hiPSC lines.

As the cells differentiate into lung endoderm (stage 2), early lung endoderm markers such as *NKX2-1*, *SOX2*, *SOX9* and *FOXP2* are upregulated (**Fig. 3c**), whereas low levels of other anterior foregut genes may still be expressed (*HNF4*, *PDX1*, *FOXP1* and *PAX9*). No expression of the forebrain marker *PAX6* should be detected. Upregulation of *PAX6* and *NKX2-1* may suggest contamination with ectoderm-derived forebrain lineage in the culture. Addition of ventralizing factors and factors that promote epithelial differentiation during embryonic lung development, such as FGF7, FGF10, BMP4 and FGF18, results in the upregulation of lung epithelial cell genes, although at lower levels relative to tracheal and lung tissues (**Fig. 5c**). Compared with our previously published differentiation method<sup>39</sup>, improving DE generation and consequently lung endoderm differentiation augments the expression of genes associated with mucus-producing cells. Importantly, greater *CFTR* expression is also observed in these cultures. Morphologically, the cells appear cuboidal, resembling an epithelium (**Fig. 5a,b**). Expansion of the immature lung cells may result in multilayer cell growth (resembling a pseudostratified epithelium, **Fig. 6a**). By stage 4, the level of upregulated gene expression for many of the proximal epithelial cell types is comparable to our previous protocol (**Fig. 6b**). By the final stage of differentiation, after 3 weeks in ALI, the expression of proximal epithelial genes is slightly greater; however, *CFTR* is greater by tenfold (**Fig. 7a**).

Most genes associated with distal epithelia are not detectable at any stage of the differentiation. Although a greater expression level of *CFTR*-expressing epithelia is generated, this modified protocol does not eliminate possible contamination of nonlung endoderm-derived cells such as the pancreas, liver and thyroid. Methods to purify the cells are needed and should be the aim of future studies. Despite the low levels of contaminating nonlung cells, robust *CFTR* activity can still be measured by iodide efflux assay (**Fig. 7b**), with cells from the CA1 hESC line abundantly expressing the mature wild-type glycosylated form of the protein comparable to our previous differentiation protocol (**Fig. 7c**). To determine whether *CFTR*



function could be functionally rescued in CF iPSC-derived epithelial cells, the cells were treated with 3  $\mu$ M VX-809 for 48 h at 37 °C. One CF line (GM00997) with F508del showed functional CFTR rescue compared with nontreated cells, as measured by iodide efflux assay (Fig. 7b). We plan to use this protocol to study the CFTR function of more CF mutant cell lines.

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**AUTHOR CONTRIBUTIONS** A.P.W., J.R. and C.E.B. conceived the study and the experimental design. A.P.W. performed and analyzed the experiments and wrote the manuscript. J.G., S.C. and S.X. performed the experiments and helped in preparing manuscript figures. All authors edited and approved the final manuscript.

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- Thomson, J.A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147 (1998).
- Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663–676 (2006).
- Zwi, L. *et al.* Cardiomyocyte differentiation of human induced pluripotent stem cells. *Circulation* **120**, 1513–1523 (2009).
- Keirstead, H.S. Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. *J. Neurosci.* **25**, 4694–4705 (2005).
- Soldner, F. *et al.* Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. *Cell* **136**, 964–977 (2009).
- Phillips, M.J. *et al.* Modeling human retinal development with patient-specific induced pluripotent stem cells reveals multiple roles for visual system homeobox 2. *Stem Cells* **32**, 1480–1492 (2014).
- Cai, J. *et al.* Directed differentiation of human embryonic stem cells into functional hepatic cells. *Hepatology* **45**, 1229–1239 (2007).
- Spence, J.R. *et al.* Directed differentiation of human pluripotent stem cells into intestinal tissue *in vitro*. *Nature* **470**, 105–109 (2010).
- Nostro, M.C. *et al.* Stage-specific signaling through TGF family members and WNT regulates patterning and pancreatic specification of human pluripotent stem cells. *Development* **138**, 861–871 (2011).
- Selekman, J.A., Grundl, N.J., Kolz, J.M. & Palecek, S.P. Efficient generation of functional epithelial and epidermal cells from human pluripotent stem cells under defined conditions. *Tissue Eng. Part C Methods* **19**, 949–960 (2013).
- Rezania, A. *et al.* Maturation of human embryonic stem cell-derived pancreatic progenitors into functional islets capable of treating pre-existing diabetes in mice. *Diabetes* **61**, 2016–2029 (2012).
- Orlova, V.V. *et al.* Generation, expansion and functional analysis of endothelial cells and pericytes derived from human pluripotent stem cells. *Nat. Protoc.* **9**, 1514–1531 (2014).
- Shcheglovitov, A. *et al.* SHANK3 and IGF1 restore synaptic deficits in neurons from 22q13 deletion syndrome patients. *Nature* **503**, 267–271 (2013).
- Yaghi, A., Zaman, A. & Dolovich, M. Primary human bronchial epithelial cells grown from explants. *J. Vis. Exp.* [doi.org/10.3791/1789](https://doi.org/10.3791/1789) (2010).
- de Jong, P.M., van Sterkenburg, M.A., Kempenaar, J.A., Dijkman, J.H. & Ponc, M. Serial culturing of human bronchial epithelial cells derived from biopsies. *In vitro Cell. Dev. Biol. Anim.* **29A**, 379–387 (1993).
- Forrest, I.A. *et al.* Primary airway epithelial cell culture from lung transplant recipients. *Eur. Resp. J.* **26**, 1080–1085 (2005).
- Zhao, L., Yee, M. & O'Reilly, M.A. Transdifferentiation of alveolar epithelial type II to type I cells is controlled by opposing TGF- $\beta$  and BMP signaling. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **305**, L409–L418 (2013).
- Kasai, H., Allen, J.T., Mason, R.M., Kamimura, T. & Zhang, Z. TGF- $\beta$ 1 induces human alveolar epithelial to mesenchymal cell transition (EMT). *Resp. Res.* **6**, 56 (2005).
- Warshamana, G.S., Corti, M. & Brody, A.R. TNF- $\alpha$ , PDGF, and TGF- $\beta$ 1 expression by primary mouse bronchiolar-alveolar epithelial and mesenchymal cells: TNF- $\alpha$  induces TGF- $\beta$ 1. *Exp. Mol. Pathol.* **71**, 13–33 (2001).
- Tanjore, H. *et al.* Alveolar epithelial cells undergo epithelial-to-mesenchymal transition in response to endoplasmic reticulum stress. *J. Biol. Chem.* **286**, 30972–30980 (2011).
- Vaughan, M.B., Ramirez, R.D., Wright, W.E., Minna, J.D. & Shay, J.W. A three-dimensional model of differentiation of immortalized human bronchial epithelial cells. *Differentiation* **74**, 141–148 (2006).
- Delgado, O. *et al.* Multipotent capacity of immortalized human bronchial epithelial cells. *PLoS ONE* **6**, e22023 (2011).
- Ott, H.C. *et al.* Regeneration and orthotopic transplantation of a bioartificial lung. *Nat. Med.* **16**, 927–933 (2010).
- Lam, E., Ramke, M., Groos, S., Warnecke, G. & Heim, A. A differentiated porcine bronchial epithelial cell culture model for studying human adenovirus tropism and virulence. *J. Virol. Methods* **178**, 117–123 (2011).
- Dupuis, A., Hamilton, D., Cole, D.E.C. & Corey, M. Cystic fibrosis birth rates in Canada: a decreasing trend since the onset of genetic testing. *J. Pediatr.* **147**, 312–315 (2005).
- Riordan, J.R. *et al.* Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* **245**, 1066–1073 (1989).
- Vanscoy, L.L. *et al.* Heritability of lung disease severity in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* **175**, 1036–1043 (2007).
- Li, W. *et al.* Understanding the population structure of North American patients with cystic fibrosis. *Clin. Genet.* **79**, 136–146 (2011).
- Samadikucharsaraei, A. & Bishop, A.E. Effects of growth factors on the differentiation of murine ESC into type II pneumocytes. *Cloning Stem Cells* **9**, 407–416 (2007).
- Samadikucharsaraei, A. *et al.* Derivation of distal airway epithelium from human embryonic stem cells. *Tissue Eng.* **12**, 867–875 (2006).
- Van Vranken, B.E. *et al.* Coculture of embryonic stem cells with pulmonary mesenchyme: a microenvironment that promotes differentiation of pulmonary epithelium. *Tissue Eng.* **11**, 1177–1187 (2005).
- Ali, N.N. *et al.* Derivation of type II alveolar epithelial cells from murine embryonic stem cells. *Tissue Eng.* **8**, 541–550 (2002).
- Roszell, B. *et al.* Efficient derivation of alveolar type II cells from embryonic stem cells for *in vivo* application. *Tissue Eng. Part A* **15**, 3351–3365 (2009).
- Rippon, H.J. *et al.* Embryonic stem cells as a source of pulmonary epithelium *in vitro* and *in vivo*. *Proc. Am. Thorac. Soc.* **5**, 717–722 (2008).
- Coraux, C. Embryonic stem cells generate airway epithelial tissue. *Am. J. Respir. Cell Mol. Biol.* **32**, 87–92 (2004).
- Ameri, J. *et al.* FGF2 specifies hESC-derived definitive endoderm into foregut/midgut cell lineages in a concentration-dependent manner. *Stem Cells* **28**, 45–56 (2009).
- Van Haute, L., De Block, G., Liebaers, I., Sermon, K. & De Rycke, M. Generation of lung epithelial-like tissue from human embryonic stem cells. *Resp. Res.* **10**, 105 (2009).
- Wang, D., Haviland, D.L., Burns, A.R., Zsigmond, E. & Wetsel, R.A. A pure population of lung alveolar epithelial type II cells derived from human embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **104**, 4449–4454 (2007).
- Wong, A.P. *et al.* Directed differentiation of human pluripotent stem cells into mature airway epithelia expressing functional CFTR protein. *Nat. Biotechnol.* **30**, 876–882 (2012).
- Huang, S.X.L. *et al.* Efficient generation of lung and airway epithelial cells from human pluripotent stem cells. *Nat. Biotechnol.* **32**, 84–91 (2014).
- Firth, A.L. *et al.* Generation of multiciliated cells in functional airway epithelia from human induced pluripotent stem cells. *Proc. Natl. Acad. Sci. USA* **111**, E1723–E1730 (2014).
- Ghaedi, M. *et al.* Human iPSC cell-derived alveolar epithelium repopulates lung extracellular matrix. *J. Clin. Invest.* **123**, 4950–4962 (2013).
- Serls, A.E., Doherty, S., Parvatiyar, P., Wells, J.M. & Deutsch, G.H. Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung. *Development* **132**, 35–47 (2005).
- Bellusci, S. *et al.* Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. *Development* **124**, 53–63 (1997).
- Kim, S.K. & Melton, D.A. Pancreas development is promoted by cyclopamine, a hedgehog signaling inhibitor. *Proc. Natl. Acad. Sci. USA* **95**, 13036–13041 (1998).
- Green, M.D. *et al.* Generation of anterior foregut endoderm from human embryonic and induced pluripotent stem cells. *Nat. Biotechnol.* **29**, 267–272 (2011).