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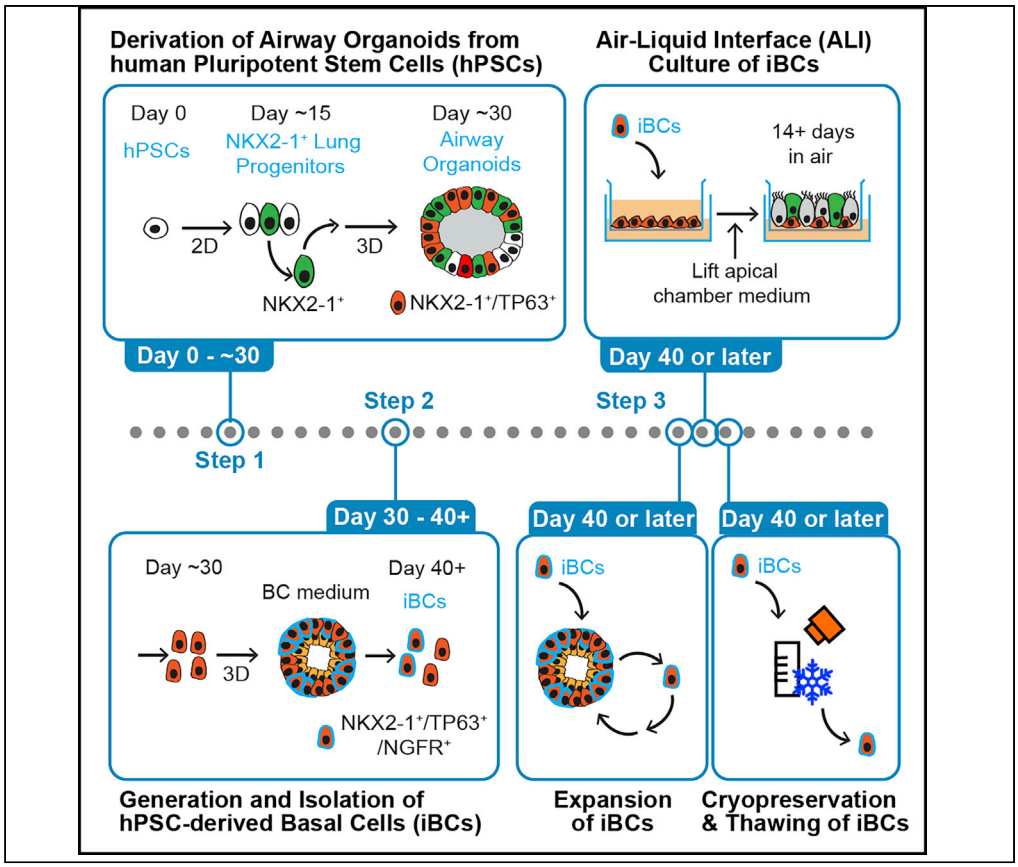
Differentiation of human pluripotent stem cells into functional airway basal stem cells



Airway basal cells play an essential role in the maintenance of the airway epithelium. Here, we provide a detailed directed differentiation protocol to generate ''induced basal cells (iBCs)'' from human pluripotent stem cells. iBCs recapitulate biological and functional properties of airway basal cells including mucociliary differentiation *in vitro* or *in vivo* in tracheal xenografts, facilitating the study of inherited and acquired diseases of the airway, as well as potential use in regenerative medicine.

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Highlights

This protocol enables differentiation of human iPSCs into airway basal cells (iBCs)

The derived iBCs can give rise to well-differentiated airway epithelium

Expansion and cryopreservation of iBCs is possible without loss of BC characteristics

This protocol is applicable to both reporter and non-reporter iPSCs

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Differentiation of human pluripotent stem cells into functional airway basal stem cells

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SUMMARY

Airway basal cells play an essential role in the maintenance of the airway epithelium. Here, we provide a detailed directed differentiation protocol to generate ''induced basal cells (iBCs)'' from human pluripotent stem cells. iBCs recapitulate biological and functional properties of airway basal cells including mucociliary differentiation *in vitro* or *in vivo* in tracheal xenografts, facilitating the study of inherited and acquired diseases of the airway, as well as potential use in regenerative medicine.

For complete details on the use and execution of this protocol, please refer to Hawkins et al. (2021).

BEFORE YOU BEGIN

Depending on the institution, the use of human pluripotent stem cells (hPSCs), including induced pluripotent stem cells (iPSCs) and embryonic stem cell (ESCs), may require prior approvals (for example from an Institutional Stem Cell Research Oversight committee).

Standard cell culture procedures and equipment, including sterile handling in the biosafety cabinets, and humidified incubators are required for maintenance and differentiation of hPSC lines. A humidified incubator is utilized at 37°C and 5% CO_2 (without regulating O_2 level) in all steps of this protocol including cell culture, Matrigel gelification, etc. hPSC lines should be tested for the expression of pluripotency markers, have a confirmed normal karyotype, and be tested to exclude mycoplasma contamination prior to use.

The directed differentiation protocol to generate iBCs from hPSCs, follows the major developmental stages from pluripotency to endoderm induction, anterior foregut patterning, lung specification and subsequently adopting an airway basal cells (BC) fate. The first identifiable lung progenitors emerge from anterior foregut endoderm as developmentally immature cells expressing the transcription factor NK2 homeobox 1 (NKX2-1). During proximal airway epithelial patterning NKX2-1$^+$ cells upregulate expression of the key basal cell transcription factor Tumor Protein 63 (TP63) and subsequently adopt an airway basal cell program. To trace and purify the targeted cell population in each step during differentiation protocol from hPSC, we engineered an iPSC line from a healthy individual (''BU3'') carrying a dual fluorescence reporter system to identify lung progenitors (*NKX2-1-GFP*)

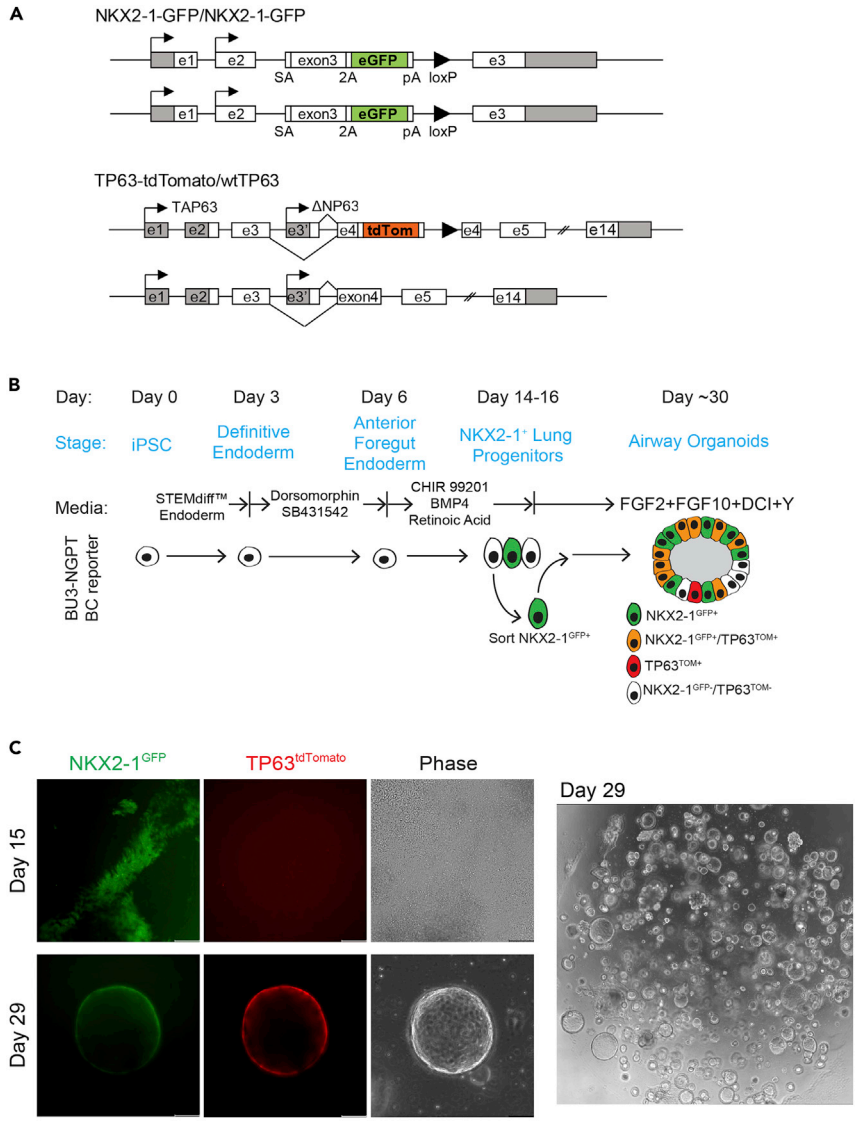

Figure 1. Direct differentiation of hPSC-derived airway epithelial organoids from basal cell reporter iPSCs, BU3-NGPT iPSCs

(A) Genomic structure of the basal cell reporter in BU3-NGPT iPSCs. eGFP gene is integrated in both alleles of *NKX2-1* and tdTomato gene is integrated in one allele of *TP63*.

(B) Schematic of the airway-directed differentiation protocol detailed in McCauley et al. 2018. Predicted fluorescence protein expression patterns from the basal cell reporter are shown in color.

(C) Representative image demonstrating GFP and tdTomato fluorescence expression in BU3-NGPT on day 15 (upper panels) or day 29 (lower panels) of differentiation. Scale bar = 100 μm Representative BU3-NGPT culture in one Matrigel droplet on day 29 of differentiation (10× magnification, 6 × 6 tile scan).

and subsequently airway progenitors (*TP63-tdTomato*; hereafter "BU3-NGPT") (Figure 1A). In this protocol we provide a detailed description of the directed differentiation strategy with the optimized conditions for this iPSC line. We have successfully applied this protocol to non-reporter iPSCs, including from iPSCs generated from individuals with genetic lung diseases, using flow cytometry sorting methodology based on cell surface markers to enrich for the desired cell population at two key steps in the directed differentiation. This alternative procedure is detailed in parallel. The iBCs generated via either approach establish well-differentiated airway epithelium composed of specialized cell types such as ciliated, secretory and BC in standard air-liquid interface (ALI) cultures.

We demonstrated the feasibility of using this airway epithelium to model three airway disorders: (1) mucus cell metaplasia in asthma; (2) abnormal ion-flux seen in Cystic fibrosis (CF); and (3) defective ciliary beating seen in Primary ciliary dyskinesia (PCD) (Hawkins et al., 2021) as examples of the potential applications of this protocol.

This protocol details all stages of the directed differentiation process, however focuses on the steps from derivation of airway progenitors in hPSC-derived airway epithelial organoids, the subsequent maturation, isolation, and expansion of iBC, and finally the differentiation of iBCs into pseudostratified, airway epithelium through ALI culture. We include methods that describe the cryopreservation and thawing protocols for iBCs. Although we summarize the protocol to obtain hPSC-derived airway epithelial organoids below (Figure 1B), we strongly recommend referring to the detailed step-by-step protocol as well as troubleshooting previously described (McCauley et al., 2018) before you begin.

Differentiation to definitive endoderm

Timing: 4 days

The BU3-NGPT iPSC line is cultured with StemFlex medium on hESC-qualified Matrigel. We have successfully differentiated multiple hPSCs cultured with mTeSR1 medium on hESC-qualified Matrigel. hPSCs maintained in culture are passaged 3–5 days prior to differentiation.

1. **Day -1** When the PSCs culture reaches approximately 60% confluent, dissociate and plate iPSCs (the following volumes and cell numbers are referred to one well of a 6 well culture plate, adjust accordingly)
   a. Coat as many wells as required with hESC-qualified Matrigel for at least 1 h in the cell culture hood (approx. 22°C) or in a humidified incubator (follow the manufacturer's instructions, https://ecatalog.corning.com/life-sciences/b2c/US/en/Surfaces/Extracellular-Matrices-ECMs/Corning®-Matrigel®-Matrix/p/354277, to dilute the Matrigel to the correct concentration as this is dependent on the lot)
   b. Aspirate the culture medium and wash the cells with DMEM/F-12 (1 mL/well) warmed to room temperature (approx. 22°C).
   c. Incubate cells with 1 mL/well of Accutase at 37°C, for 5 min, or with 1 mL/well of Gentle Cell Dissociation Reagent at 37°C, for 7–9 min.
   d. Pipette gently to dissociate into single cells, dilute with DMEM/F-12 (1 mL or more/well), and count. Aliquot cell suspension as needed in the following step and centrifuge at 300 g for 5 min.
   e. Plate 2 × 10^6 cells/well with 2 mL/well of mTeSR1 medium supplemented with 10 μM Y-27632
2. **Day 0** Initiate the differentiation into definitive endoderm using STEMdiff Definitive Endoderm Kit (https://www.stemcell.com/stemdiff-definitive-endoderm-kit.html).
   a. 24 h post-plating, wash the cells twice with DMEM/F-12 (1 mL/well).
   b. Add 2 mL/well of STEMdiff Definitive Endoderm medium with supplements MR and CJ (10 μL of each supplement in 980 μL basal medium)
3. **Day 1 (24 h)** change to 2 mL/well STEMdiff Definitive Endoderm medium with supplement CJ only (10 μL of supplement in 990 μL basal medium).
4. **Day 2 (48 h)** feed with 2 mL/well STEMdiff Definitive Endoderm medium with supplement CJ as on Day 1. Keep feeding every 24 h with this medium until definitive endoderm differentiation step is complete.

⚠ CRITICAL: The hours of the optimal endoderm induction for lung differentiation need to be optimized for each line, generally from 54 h through 72 h. For BU3-NGPT, 56–60 h. The optimal timing can be tested by harvesting the cells at multiple time points and staining for

CXCR4 and c-KIT. We typically choose the earliest time point at which >90% of cells co-express C-KIT and CXCR4. Troubleshooting 1

Note: Any culture medium as well as a medium (such as DMEM/F-12) to wash cells or to dilute dissociation solution should be warmed to approx. 22°C (room temperature), unless specified. The centrifuge is also set to approx. 22°C.

Differentiation to anterior foregut endoderm

Timing: 72 h

5. Day 3 or 4 (depending on the stopping point of step 4) Detach and re-plate the definitive endoderm
   a. Coat as many wells as required with hESC-qualified Matrigel as above.
   b. Aspirate the culture medium and wash twice with DMEM/F-12 (1 mL/well).
   c. Add 1 mL/well of Gentle Cell Dissociation Reagent and incubate 2–4 min at by monitoring the cells condition under the microscope until cells start to appear rounded and cell-cell contact is reduced.
   d. Aspirate carefully only the Gentle Cell Dissociation Reagent (do not disturb cells) and add 1 mL/well of DS/SB medium (see Table in Materials and Equipment) + 10 μM Y-27632. Gently pipet with 1000 μL pipette or serological pipette to detach the cells as small clumps, approximately 10 cells per clump.
   e. Transfer the clumps into a sterile 15 mL or 50 mL conical tube and dilute the clumps with DS/SB medium + 10 μM Y-27632 to the desired volume.
   f. Plate 2 mL/well of cell suspension.
6. after 24 h Feed with DS/SB medium and culture for a further 48 h.

⚠ CRITICAL: The passaging dilution at steps 5e and 5f needs to be optimized for each line as it affects the efficiency of NKX2-1$^+$ lung specification. The optimal plating dilution is that which maximizes the percentage of NKX2-1$^+$ lung progenitor at Day 14–16 of differentiation. For BU3-NGPT, 1:5 to 1:6 (1 well to 5–6 wells).

Differentiation to NKX2-1$^+$ lung progenitors

Timing: ~10 days

7. ~ Day 6 Change the medium to CBRA (see Table in Materials and Equipment). Proceed with feeding at least every 48 h or more frequently until Day 14–16 of differentiation

⚠ CRITICAL: It is important to only add retinoic acid (RA) (100 nM final concentration) to make the complete CBRA medium (see Table in Materials and Equipment) at the time of feeding. Protect both RA and CBRA medium from light. The optimal feeding schedule can be varied for each iPSC line between 24 and 48 h. Some iPSC lines have faster metabolism indicated by yellow color of medium when supplemented with phenol red and require more frequent feeding. For BU3NGPT: every ~36 h.

Differentiation of NKX2-1$^+$ lung progenitors into airway epithelial organoids

Timing: ~15 days

8. Day 14–16 Detach the cells and sort NKX2.1$^+$ cells using either reporter or surface marker approaches.
   a. Aspirate the culture medium and wash twice with DMEM/F-12.

b. Remove DMEM/F-12 from no more than 2 wells at a time and use a 10 μL pipet tip to etch 3 to 4 scrapes both vertically and horizontally across the monolayer in a well of 6 well plate (this facilitates the cell detachment).
c. Immediately add 1 mL/well of Accutase and incubate at 37°C for 10 min.
d. Gently pipet with 1000 μL pipet several time to lift the cells as sheets and incubate 5–10 additional minutes.
e. Gently pipet to detach and dissociate cells to 2–3 cells per aggregate to single cells and then collect the cells into DMEM/F-12 (1 mL or more/well) to dilute the Accutase. **Troubleshooting 2**
f. Pass the cell suspension through a 40 μm cell strainer and count.

Note: For **BU3-NGPT** follow step **g**, i, j, k, and for **Non reporter iPSC** follow step **h**, i, j, k.

g. ***BU3-NGPT:*** sort GFP^+ cells as $NKX2\text{-}1^+$ cells through Fluorescence-Activated Cell Sorting (FACS).
   i. Resuspend in FACS buffer (see Table in Materials and Equipment) + 10 μM Y-27632 (5 to 10 million cells/mL) + live cell dye (i.e., Calcein Blue) or dead cell dye (i.e., Propidium Iodide).
   ii. Sort GFP^+ live cells.
h. ***Non reporter iPSC:*** Enrich $NKX2\text{-}1^+$ cells through FACS, targeting cell surface markers.
   i. Resuspend in FACS buffer+ anti-CPM antibody (1:200) (Gotoh et al., 2014) (10 million cells/200 μL) and incubate 15 min at 4°C, or conjugated anti-CD47 (1:200) and anti-CD26 (1:200) antibodies (Hawkins et al., 2017) and incubate 30 min at 4°C.
   ii. (Per CPM antibody staining) Wash and incubate with secondary antibody (1:500) 15 min at 4°C.
   iii. Wash and resuspend into FACS buffer+ 10 μM Y-27632 (5 to 10 million cells/mL) + live or dead cell dye.
   iv. Sort CPM^{high} or $CD47^{high}/CD26^{-}$ live cells.
i. Collect sorted cells in FACS buffer+ 10 μM Y-27632 and count.
j. Resuspend 400 cells/μL of growth factor-reduced Matrigel, undiluted and ice-cold (keep cells and Matrigel lower than 10°C, for example on ice, to avoid gelification). Plate 50 μL/well as a droplet at the center of the well in a 12 well plate.
k. Incubate the droplets in a humidified incubator for 10–20 minutes before adding 1 mL/well of F2+10+DCI+Y medium (see Table in Materials and Equipment).

9. **Up to day 28–35** Feed with F2+10+DCI+Y medium every three days.

Optional: As an alternative of Accutase at steps 8c–8e, add 0.05% Trypsin (1 mL/well) at 37°C for a total of 15 minutes, pipetting several times after 10 minutes, add an equal volume of DMEM/F-12 with 10% fetal bovine serum before proceeding to step 8f.

Note: On day ~15, the majority of $NKX2\text{-}1^{GFP+}$ cells are expected to be $TP63^{tdTomato}$ negative. By day 28–35 in F2+10+DCI+Y medium, cells have formed epithelial spheres readily identifiable in the Matrigel droplets. At this stage, $TP63^{tdTomato+}$ cells emerge within $NKX2\text{-}1^{GFP+}$ cell population (Figure 1C).

Note: The size of the droplets is typically between 20–50 μL.

Note: All sorting steps have been performed on either BD FACSMelody™ Cell Sorter or MoFlo Astrios Cell Sorter. On BD FACSMelody™ Cell Sorter, Propidium Iodide was used to remove dead cells and cells were sorted using 100 μm nozzle at ~4000–6000 of the event rate in FACSChorus software. On MoFlo Astrios Cell Sorter, Calcein Blue was used to select the live cells and the cells were sorted using a 100 μm nozzle 30 psi at a flow speed of 0.5–0.7 psi analog in the Summit software.

Note: General laboratory consumables such as serological pipettes (2 mL, 5 mL, 10 mL, 25 mL, 50 mL), pipet tips (10 μL, 20 μL, 200 μL and 1000 μL), aspirating pipettes, and conical centrifuge tubes (1.5 mL, 2 mL, 15 mL, 50 mL) are also required.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-Acetylated Tubulin (ACT) (6-11B-1)	MilliporeSigma	Cat.# T7451, RRID: AB_609894
Mouse Monoclonal Anti-TP63 (4A4)	Biocare	Cat.# CM163A, RRID: AB_10582730
Rabbit monoclonal anti-MUC5AC (E309I)	Cell Signaling Technology	Cat.# 61193, RRID: AB_2799603
Rabbit monoclonal anti-p75NTR/NGFR (D4B3)	Cell Signaling Technology	Cat.# 8238, RRID: AB_10839265
Mouse monoclonal anti-CC10 (E-11) (SCGB1A1)	Santa Cruz	Cat.# Sc-365992, RRID: AB_10915481
Rabbit monoclonal anti-NKX2-1 (EP1584Y)	Abcam	Cat.# ab76013, RRID: AB_1310784
Mouse monoclonal anti-Carboxypeptidase M (CPM)	Fujifilm	Cat.# 014-27501, RRID:AB_2801482
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 555	Invitrogen	Cat.# A32794, RRID:AB_2762834
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Invitrogen	Cat.# A-21202, RRID:AB_141607
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 647	Invitrogen	Cat.# A32787, RRID:AB_2762830
APC-mouse monoclonal anti-CD184/CXCR4 (12G5)	BioLegend	Cat.# 306510, RRID:AB_314616
PE-mouse monoclonal anti-CD117/c-Kit (104D2)	BioLegend	Cat.# 313204, RRID:AB_314983
APC-mouse monoclonal anti-CD47 (CC2C6)	BioLegend	Cat.# 323123, RRID: AB_2716202
PE-mouse monoclonal anti-CD26 (BA5b)	BioLegend	Cat.# 302705, RRID: AB_314289
APC/Fire750-mouse monoclonal anti-EpCAM (9C4)	BioLegend	Cat.# 324234: RRID: AB_2629703
APC-mouse monoclonal anti-human CD271/NGFR (ME20.4)	BioLegend	Cat.# 345108; RRID: AB_10645515
Mouse IgG1kappa isotype control, APC-conjugated	BioLegend	Cat.# 400122; RRID: AB_326443
Mouse IgG1 isotype control, PE-conjugated	BioLegend	Cat.# 400113; RRID: AB_326435
Mouse IgG1 isotype control, PerCP/Cy5.5-conjugated	BioLegend	Cat.# 400149; RRID: AB_893680
Chemicals, peptides, and recombinant proteins		
SB431542	Tocris	Cat.# 1614
Dorsomorphin	Stemgent	Cat.# 04-0024
CHIR99021	Tocris	Cat.# 4423
Recombinant Human BMP4	R&D Systems	Cat.# 314-BP
Retinoic acid	Sigma	Cat.# R2625
Y-27632 dihydrochloride	Tocris	Cat.# 1254
Recombinant Human FGF10	R&D Systems	Cat.# 345-FG-025
Recombinant Human FGF2	R&D Systems	Cat.# 233-FB
Dexamethasone	Sigma	Cat.# D4902
8-Bromoadenosine 30,50-cyclic monophosphate sodium salt (cAMP)	Sigma	Cat.# B7880
3-Isobutyl-1-methylxanthine (IBMX)	Sigma	Cat.# I5879
A83-01	Thermo Fisher Scientific	Cat.# 293910
DMH1	Thermo Fisher Scientific	Cat.# 412610
L-Ascorbic acid	Sigma	Cat# A4544
1-thioglycerol (MTG)	Sigma	Cat.# M6145
Propidium Iodide (PI) solution	BioLegend	Cat.# 421301
Calcein Blue, AM	Themo Fisher Scientific	Cat# C1429

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Sc-RNA-Seq data of iBCs and iBC-derived ALI and primary HBECs and HBEC-ALI	Kotton Lab/Hawkins Lab	GEO: GSE142246
Experimental models: Cell lines		
Human: Normal donor iPSC line targeted with NKX2-1GFP and P63tdTomato (BU3-NGPT)	Kotton Lab (Hawkins et al., 2021)	www.bumc.bu.edu/stemcells
Software and algorithms		
ImageJ	National Institutes of Health	https://Imagej.nih.gov/ij/
FlowJo	Becton, Dickinson & Company	https://www.Flowjo.com
FACSChorus	Becton, Dickinson & Company	N/A
Summit	Beckman Coulter	N/A
Other		
mTeSR1	STEMCELL Technologies	Cat.# 05850
Stemdiff Definitive Endoderm Kit	STEMCELL Technologies	Cat.# 05110
PneumaCult ALI Medium	STEMCELL Technologies	Cat.# 05001
PneumaCult ExPlus Medium	STEMCELL Technologies	Cat.# 05040
StemFlex medium	Thermo Fisher Scientific	Cat.# A3349401
Matrigel Growth Factor Reduced	Corning	Cat.# 356230
Matrigel hESC-Qualified Matrix	Corning	Cat.# 354277
6.5 mm Transwell® with 0.4 μm Pore Polyester Membrane Insert	Corning	Cat# 3470
ReLeSR	STEMCELL Technologies	Cat# 05873
Dispase (5U/mL)	STEMCELL Technologies	Cat# 07913
Accutase	STEMCELL Technologies	Cat# 07920
Gentle Cell Dissociation Reagent	STEMCELL Technologies	Cat# 07174
0.05% trypsin	Gibco	Cat# 25-300-062
GlutaMAX Supplement (100×)	Gibco	Cat# 35050
Fetal Bovine Serum (FBS)	Gibco	Cat# 10082139
Bovine Albumin Fraction V, 7.5% solution	Gibco	Cat# 15260-037
B27 Supplement (50×), serum free	Gibco	Cat# 17504044
N-2 Supplement (100×)	Gibco	Cat# 17502048
HEPES (1M)	Gibco	Cat# 15630080
DMEM/F12	Invitrogen	Cat# 11320033
Iscove's Modified Dulbecco's Medium (IMDM)	Invitrogen	Cat# 12200-036
Ham's F12	Invitrogen	Cat# 11765-054
Ultrapure 0.5M EDTA, pH8.0	Invitrogen	Cat# 15575020
Primocin	Invitrogen	Cat# ant-pm-2
Penicilin/Streptomycin (10,000U/mL)	Invitrogen	Cat# 15140122
Falcon 40 μm Cell strainer	Corning	Cat# 352340
Falcon 6 well culture plate	Corning	Cat# 353046
Falcon 12 well culture plate	Corning	Cat# 353043
CryoStor CS10	BioLife Solutions	Cat#: 210102
NutriFreez D10	Biological Industries	Cat# SKU: 05-713-1A
M.O.M. (Mouse on Mouse) Blocking Reagent	Vector Laboratories	Cat# MKB-2213-1
Heracell 150i CO_2 Incubator	Thermo Scientific	50116048
BD FACSMelody (lasers: 488 nm, 561 nm, and 640 nm)	BD Biosciences	N/A
MoFlo Astrios (lasers: 355 nm, 405 nm, 488 nm, 561 nm, and 640 nm)	Beckman Coulter	N/A
Leica DMi8 microscope	Leica Microsystems	N/A
Nikon Eclipse Ni-E microscope	Nikon	N/A
Cytospin 4	Thermo Scientific	Cat# A78300002
Centrifuge 5810R	Eppendorf	Cat# 022625101
Centrifuge 5424R	Eppendorf	Cat# 5404000014

MATERIALS AND EQUIPMENT

cSFDM: complete serum-free differentiation medium		
Reagent	Final concentration	Amount
IMDM	n/a	362.0 mL
Ham's F-12	n/a	120.7 mL
B27 with retinoic acid (50×)	0.5×	5 mL
N2 supplement (100×)	0.5×	2.5 mL
Ascorbic acid (50 mg/mL)	50 μg/mL	0.5 mL
Monothioglycerol	500 μg/mL	19.5 μL
Bovine Albumin Fraction V, 7.5%	0.05625%	3.75 mL
Glutamax (100×)	1×	5 mL
Antibiotics (Primocin, 50 mg/mL)	50 μg/mL	0.5 mL
Total	**n/a**	**500 mL**
Store at 4°C for up to 1 month, away from light		

Note: As an alternative of Primocin, use Penicilin/Streptomycin at 100U/mL.

DS/SB medium		
Reagent	Final concentration	Amount
cSFDM	n/a	998 μL
Dorsomorphin (DS) (2 mM)	2 μM	1 μL
SB431542 (SB) (10 mM)	10 μM	1 μL
Total	**n/a**	**1 mL**
Store at 4°C for up to 1 week		

CBRA medium		
Reagent	Final concentration	Amount
cSFDM	n/a	997 μL
CHIR99021 (3 mM)	3 μM	1 μL
rhBMP4 (10 μg/mL)	10 ng/mL	1 μL
Retinoic acid (100 μM)	100 nM	1 μL
Total	**n/a**	**1 mL**
Store cSFDM supplemented with CHIR99021 and rhBMP4 at 4°C for up to 1 week		

Note: Add retinoic acid (RA) to make the complete CBRA medium at the time of feeding. Protect both RA and CBRA medium from light.

FGF2+10+DCI+Y		
Reagent	Final concentration	Amount
cSFDM	n/a	44.825 mL
cAMP/IBMX (10×)	1×	5 mL
rhFGF2 (250 μg/mL)	250 ng/mL	50 μL
rhFGF10 (100 μg/mL)	100 ng/mL	50 μL
Dexamethasone (100 μM)	50 nM	25 μL
Y-27632 (10 mM)	10 μM	50 μL
Total	**n/a**	**50 mL**
Store at 4°C for up to 2 weeks		

Note: See details in McCauley et al. 2018 to prepare and to store the media listed above.

FACS buffer		
Reagent	Final concentration	Amount
Phosphate buffered saline	n/a	475 mL
Fetal bovine serum	2%	10 mL
HEPES buffer (1M)	25 mM	12.5 mL
EDTA solution (500 mM)	2 mM	2 mL
Antibiotics (Primocin, 50 mg/mL)	1×	0.5 mL
Total	**n/a**	**500 mL**
Store at 4°C for up to 1 month		

Note: As an alternative of Primocin, use Penicilin/Streptomycin at 100U/mL.

Basal cell medium		
Reagent	Final concentration	Amount
PneumaCult ExPlus	1×	9.988 mL
A83-01 (10 mM in DMSO)	1 μM	1 μL
DMH1 (10 mM in DMSO)	1 μM	1 μL
Y-27632 (10 mM)	10 μM	10 μL
Total	**n/a**	**10 mL**
Store at 4°C for up to 1 month		

Note: Follow the manufacturer instruction to complete PneumaCult ExPlus (1×) https://www.stemcell.com/pneumacult-ex-plus-medium.html

STEP-BY-STEP METHOD DETAILS

The protocol outlined above, starting with hPSCs, is designed to generate hPSC-derived airway epithelial organoids. The following step by step method details the subsequent derivation of iBCs (starting from Day ~30 of differentiation (Figure 2A)), including their expansion, cryopreservation, and *in vitro* differentiation into airway epithelium.

Evaluation of NKX2-1$^+$/TP63$^+$ airway progenitors in hPSC-derived airway epithelial organoids

Timing: 4 h–2 days

This step evaluates how successful is the preparation of hPSC-derived airway epithelial organoids. A visual inspection may be done for BU3-NGPT with fluorescent microscopy imaging (Figure 2B). To quantify, flow cytometric analysis (Figure 2C) or preparing a cytospin followed by immunofluorescence staining (Figure 2D) should be performed. Cells in one droplet/well should be sufficient for this step. This confirmation can be performed **several days prior to the next step.** (This step is optional, but recommended)

1. Digest Matrigel to release organoids
   a. Remove media and add 0.5 mL Dispase (1U/mL) per well, dislodge Matrigel droplets mechanically using a pipette tip but without pipetting and incubate at 37°C for 30 min in a humidified incubator.

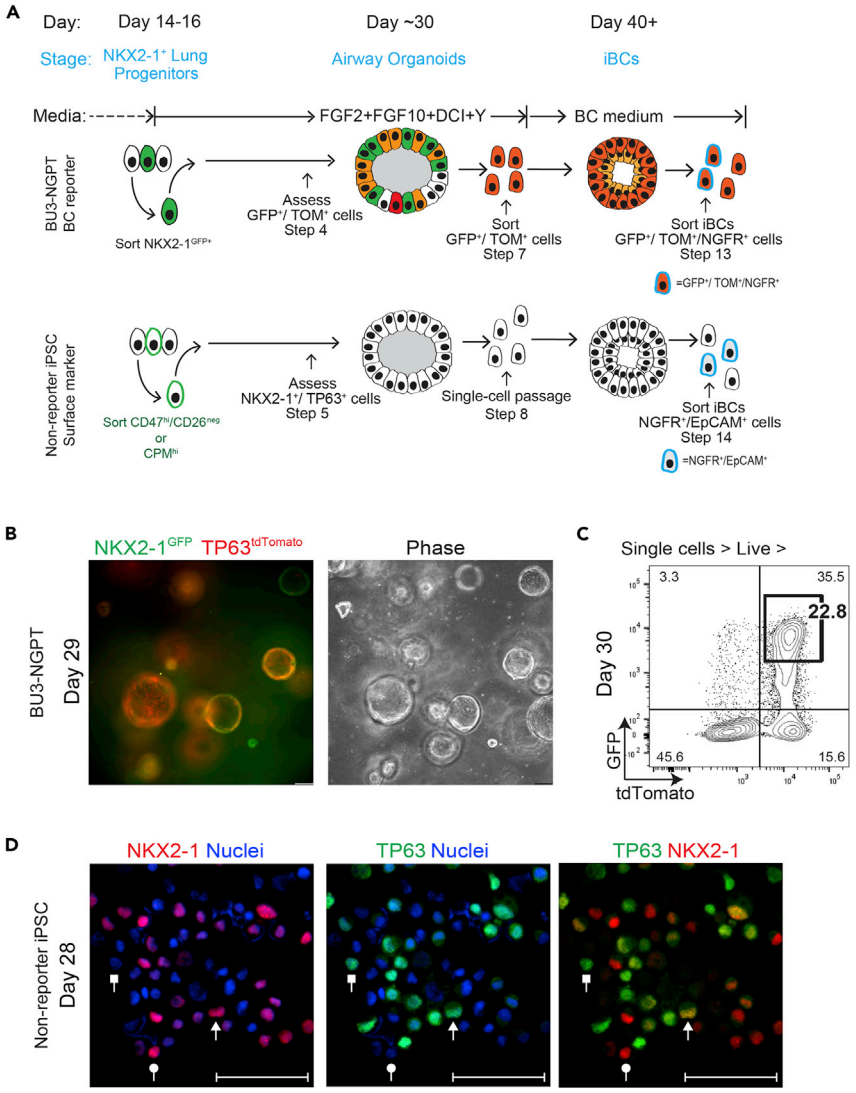

Figure 2. Generation of iBCs

(A) Schematic of iBC differentiation protocol from BU3-NGPT (top row) or non-reporter iPSCs (bottom row).

(B and C) Representative expression pattern of GFP and tdTomato via microscopic analysis on Day 29(B) or flow cytometry analysis in single live cells on Day 30(C). The suggested gating for NKX2-1^{GFP+} and TP63$^{tdTomato+}$ population is shown with bold line square.

(D) Immunolabeling of non-reporter iPSCs with antibodies against NKX2-1 and TP63 on day 28 of directed differentiation. DNA is labeled with DAPI; Scale bar = 50 μm. The round arrowhead indicates a NKX2-1 single positive cell, the square arrowhead a TP63 single positive, and the triangle arrowhead a NKX2-1 and TP63 double positive.

b. Pipette Dispase and Matrigel together several times to break Matrigel down until Matrigel is no longer visible by eye and incubate again at 37°C for approximately 30 min to 1 h in a humidified incubator. Confirm under a light microscope that there is no visible Matrigel remaining. If any, incubate an additional 15–30 min.

c. Dilute with DMEM/F-12 (1 mL or more/well) and transfer to a 15 or 50 mL conical tube and pellet organoids by centrifugation, 200–300 g, 3–5 min. Remove supernatant.

Note: Before the incubation at step 1a, Matrigel tends to stick to pipette tips. Allowing 30 minutes to incubate with Dispase before pipetting at step 1b. helps to avoid the loss of cells while dislodging the droplets accelerates their degradation.

2. Dissociate organoids to single cells
   a. Treat with 1 mL 0.05% trypsin per harvested well. Gently pipette or invert, lay the tube on its side and incubate at 37°C, 5 min.
   b. Gently invert the tube several time to mix and avoid aggregation of cells at the bottom or on the wall of the conical tube, and incubate for another 5 min. Confirm that the majority of clumps are not visible.
   c. Neutralize trypsin with equal volume of DMEM/F-12 supplemented with 10% FBS. Pellet cells by centrifuge at 300 *g* for 5 min.
   d. Treat 1 mL ReLeSR per well to further dissociate the remaining small clumps to single cells at approx. 22°C for 10 min. Occasionally invert to mix.
   e. Dilute ReLeSR with equal or greater volume of DMEM/F-12 and pass cells through a 40 μm cell strainer, and then count.

⚠ CRITICAL: Longer incubation in trypsin may be required during step 2.b, up to an additional 5 minutes. However, longer trypsinization causes significant cell death. Small clumps, several cells per aggregate, can be further dissociated by non-enzymatic dissociation reagents, such as ReLeSR, to avoid excess cell damage from enzymatic dissociation. In general, a half million to one million cells per organoid can be expected after the dissociation step.

3. Pellet cells by centrifuging at 300 *g* for 5 min. Remove supernatant.

Note: For **BU3-NGPT** follow step **4**, and for **Non reporter iPSC** follow step **5**.

4. *BU3-NGPT:* Analyze percentage of GFP and tdTomato with flow cytometry (Figure 2C)
   a. Resuspend in FACS buffer+ live or dead cell dye
   b. Analyze with flow cytometry
5. *Non reporter iPSCs:* Analyze percentage of cells expressing NKX2-1 and TP63 by cytospin and immunofluorescence staining (Figure 2D)
   a. Spin down 10^5 single cells on a charged slide mounted in a cytospin funnel at 100 *g* for 5 min.
   b. Remove the funnel and let cells dry for 30 s to 1 min.
   c. Fix the cells on the slide with 4% PFA in PBS for 15 min.
   d. Wash the cells three times with PBS.
   e. Permeabilize the cells with 0.3% triton X in PBS for 15 min.
   f. Block the sample with 2% BSA solution at approx. 22°C for 1 h.
   g. Immunolabel samples with primary antibodies against NKX2-1 (1:500) and TP63 (1:200) in 2% BSA solution approx. 16 h at 4°C or 1 h at approx. 22°C.
   h. Wash three times for 5 min with PBS.
   i. Incubate samples with secondary antibodies, Alexa488-Donkey anti-mouse IgG (1:500) and Alexa555-donkey anti-rabbit IgG (1:500) in 2% BSA solution for 1 h at approx. 22°C in dark.
   j. Wash three times for 5 min with PBS.
   k. Mount with ProLong Gold Antifade Mountant with DAPI, seal with a coverslip and cure for 24 h before imaging.
   l. Quantify the cells that are NKX2-1 and TP63 positive in their nuclei.

Pause point: PFA-fixed cells on slides can be stored in PBS at 4°C prior to start staining up to one month.

Generation of iPSC-derived basal cells (iBCs)

Timing: 10–14 days

In this step, NKX2-1^+/TP63^+ airway progenitors in hPSC-derived airway epithelial organoids are further matured into iBCs with transcriptional and functional similarity to adult primary airway

basal cells, including the expression of the surface receptor Nerve Growth Factor Receptor (NGFR).

6. ~**Day 30** Follow steps 1 through 3 described above (Evaluation of NKX2-1^{+}/TP63^{+} airway progenitors in hPSC-derived airway epithelial organoids) to obtain single cells from hPSC-derived airway epithelial organoid.

   Note: For **BU3-NGPT** follow step **7**, 9, 10, 11 and for **Non reporter iPSC** follow step **8,** 9, 10, 11.

7. *BU3-NGPT:* Sort live NKX2-1^{GFP+} cells that co-express TP63$^{tdTomato+}$ live cells (Figure 2C)
8. *Non reporter iPSCs:* Skip step 7 and move to step 9. **Troubleshooting 3**
9. Re-plate 20,000 NKX2-1^{GFP+}/TP63$^{tdTomato+}$ cells in a 25–50 μL Matrigel droplet per well of 12 well plate at 400 - 800 cells per μL of Matrigel. Place the culture plate in a humidified incubator for 10–20 min and then add 1 mL FGF2+10+DCI+Y medium per well.
10. One day after re-plating, change the culture medium to basal cell medium (see Table in Materials and Equipment).
11. Feed every 2–3 days and culture ~9 days or longer in basal cell medium.

Evaluation, isolation and expansion of iBCs

Timing: 1 day

This step describes how iBCs, the targeted cell population generated through this protocol, can be evaluated and isolated based on NGFR expression. At this stage, the organoid morphology has evolved with denser appearing spheres (Figure 3A), while the earlier culture stages contain spheres with thinner, single-cell layer, spheres (Figure 2B).

12. ~**Day 40 or later** Follow steps 1 through 3 to obtain single cells from airway epithelial organoids cultured in basal cell medium

    Note: For **BU3-NGPT** follow step **13**, 15, and for **Non reporter iPSC** follow step **14,** 15.

13. *BU3-NGPT:* Evaluate and sort iBC (Figure 3B)
    a. Resuspend single cells in FACS buffer.
    b. Stain cells with NGFR antibody (1:100) (and EpCAM antibody, 1:100) in 50 μL of FACS buffer per 1 million cells for 30 min at 4°C.
    c. Wash and resuspend in FACS buffer+ 10 μM Y-27632 (5 to 10 million cells/mL) + live or dead cell dye
    d. Sort NKX2-1^{GFP+}, TP63$^{tdTomato+}$, and NGFR^{+} (and EpCAM^{+}) population to isolate iBC.
14. *Non reporter iPSC:* Evaluate and sort iBC (Figure 3C)
    a. Resuspend single cells in FACS buffer.
    b. Stain cells with NGFR antibody (1:100) (and EpCAM antibody, 1:100) in 50 μL of FACS buffer per 1 million cells for 30 min at 4°C.
    c. Wash and resuspend into FACS buffer+ 10 μM Y-27632 (5 to 10 million cells/mL) + live or dead cell dye.
    d. Sort NGFR^{+} (and EpCAM^{+}, **Troubleshooting 4**) population to isolate iBC. **Troubleshooting 5**
15. Re-plate 20,000 sorted cells in a 25–50 μL Matrigel droplet per well of 12 well plate at 400–800 cells per μL of Matrigel. Place the culture plate in a humidified incubator for 10–20 min and then add 1 mL basal medium per well. Feed every 2–3 days.

    Note: iBCs sorted at step 13 or 14 are ready to use for experiments requiring ALI culture. If necessary iBCs can be expanded by passaging every 10–14 days by following step 15. Some PSC lines such as BU3-NGPT maintain high percentage of iBCs in the culture (>80%)

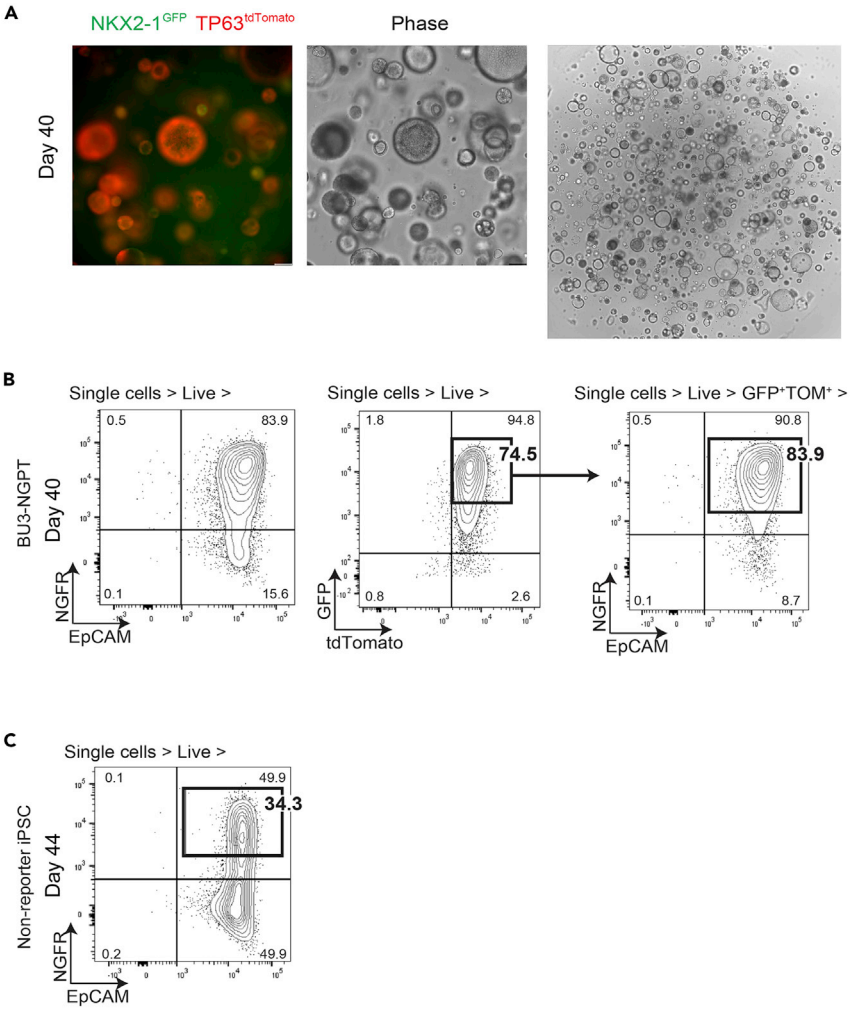

Figure 3. Examination and isolation of iBCs

(A) Representative image of organoid on day 40 of differentiation showing GFP and tdTomato expression (left panel) and its morphology (middle panel). Representative BU3-NGPT culture in one Matrigel droplet on day 40 of differentiation (Right panel, 10× magnification, 7 × 7 tile scan).

(B) Representative expression pattern of NGFR and EpCAM (left panel) or GFP and tdTomato (middle panel) in single live cells of BU3-NGPT on day 40. iBCs are gated first for NKX2-1^{GFP+} and TP63$^{tdTomato+}$ population in bold line square (middle panel) and then sorted for NGFR^{+}EpCAM^{+} population in bold line square (right panel).

(C) Representative expression pattern of NGFR and EpCAM (left panel) in single live cells of non-reporter iPSC on day 44. The suggested gating for NGFR^{+}EpCAM^{+} iBC population is shown with bold line square.

over time, allowing to simply re-plate single cells at every passaging. On the other hands, some PSC lines may require additional NGFR (and EpCAM) sorts (step 14) to enrich for iBCs and remove the secretory and to a lesser extent multiciliated cells that can differentiate from iBCs in this culture format. In particular, a high proportion of NGFR^{+} iBCs is required for ALI cultures or *in vivo* Xenograft model and thus NGFR sorting is recommended prior to transitioning to these steps.

Optional: We recommend performing a post-sort analysis of iBCs generated from non-reporter iPSC to check the purity based on NKX2-1 and TP63 co-expression. Perform cytospin and immunofluorescence staining by following step 5 with additional blocking to mask mouse antibodies used for sorting. For this purpose, use MOM (Mouse On Mouse) blocking reagent at the dilution of 2 drops in 2.5 mL PBS, 1 h at approx. 22°C, prior to step 5f.

Cryopreservation and thawing of iBC

Timing: 2 weeks

Cryopreservation of iBCs offers significant practical benefits and avoids the need to redo the lengthy differentiation protocol for every experiment. This step explains the methods for the cryopreservation and subsequent thawing of iBCs.

Note: We have tested cryopreservation at several stages of the differentiation protocol. From our experience, cryopreservation is most successful, in terms of viability, at the iBCs stage. Cryopreservation prior to ~day 40 of differentiation is not recommended.

16. ~Day 40 or later: Cryopreservation
    a. Follow steps 1 through 3 from the "Evaluation of NKX2-1$^+$/TP63$^+$ airway progenitors in hPSC-derived airway epithelial organoids" section above to obtain single cells from airway epithelial organoids cultured in basal cell medium.
    b. Resuspend single cells in basal cell medium supplemented with 10% dimethylsulfoxide (DMSO) at a density of 200,000–500,000 cells per mL and transfer to a cryovial (1 mL per vial).
    c. Place in cryostorage containers and cool in a −80°C freezer at −1°C/min for 24 h and then transfer to −150°C freezer or into liquid nitrogen for long-term storage.

Optional: If the culture contains a low percentage of iBCs (less than ~50%), sorting prior to freezing is recommended.

Optional: We have also used a proprietary freezing medium with 10% DMSO, such as CryoStor CS10 and NutriFreez D10.

17. Thawing: Thaw the cryopreserved cells using conventional cell culture methods. Briefly working quickly place the vial in a 37°C water or bead bath until almost completely thawed, transfer to a 15 mL conical and add 10 mL of DMEM/F-12 drop by drop, and then pellet by centrifugation at 300 *g*, 5 min.
18. Plate 200,000 cells in a 50 μL Matrigel droplet per well of 12 well plate at 4,000 cells per μL of density in Matrigel. Incubate in a humidified incubator for 10–20 min and then add 1 mL basal medium per well. Feed every 2–3 days.
19. 3–4 days later, dissociate organoids to single cells by following steps 1 through 3.
20. Re-plate 20,000 live cells in a 50 μL Matrigel droplet per well of 12 well plate at 400 cells per μL of Matrigel. Place the 12 well culture plate in a humidified incubator for 15–30 min and then add 1 mL basal medium per well. Feed every 2–3 days.
21. 10–14 days later, iBCs are ready to expand or to sort prior to the use for the desired experiment.

Note: Significant cell death may be observed after thawing (the survival rate can vary greatly between 10 and 70%). Therefore, we recommend plating cells at a higher density at step 18 and re-plate based on live cell count, using trypan blue staining at step 20, to further expand cells at the appropriate cell density.

Establishing air-liquid interface culture (ALI) from iBCs

Timing: 3 weeks

iBCs establish pseudostratified, well-differentiated airway epithelium through, but not limited to, *in vitro* air-liquid interface (ALI) culture or *in vivo* tracheal xenograft. ALI culture is a widely used and well-established methodology for *in vitro* airway biology research. This step explains how to establish ALI cultures from iBCs. (Figure 4A).

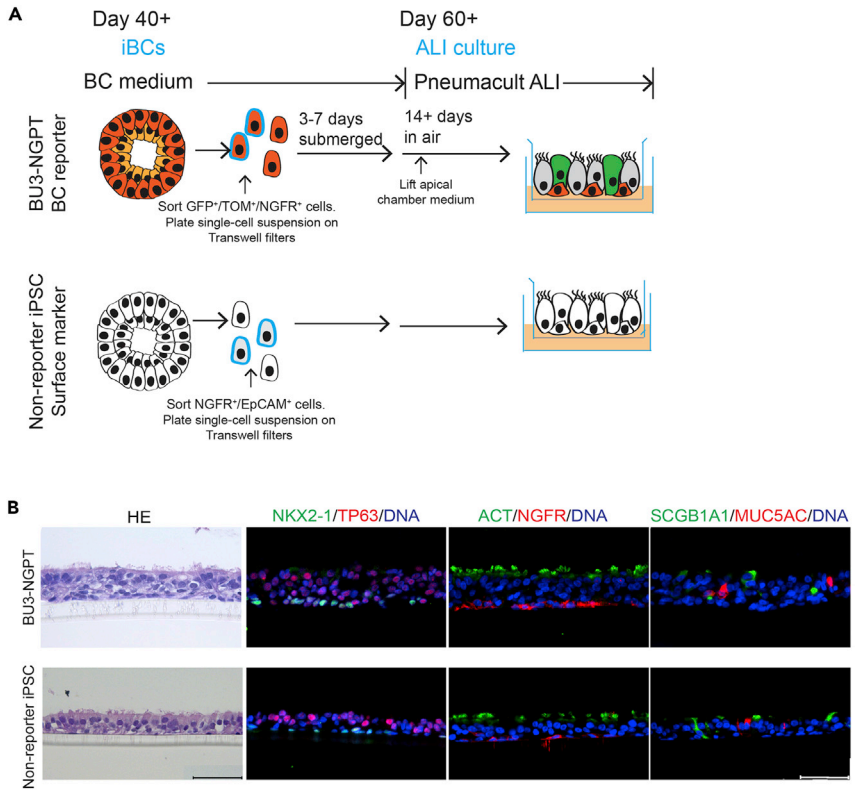

Figure 4. Establishing iBC-derived airway epithelium through *in vitro* ALI culture

(A) Schematic of ALI culture protocol with iBCs derived from BU3-NGPT (top row) or non-reporter iPSC (bottom row).

(B) Transverse section of ALI cultures derived from BU3-NGPT iBCs (top row) or non-reporter iPSC (bottom row) and stained with hematoxylin and eosin (HE) or antibodies indicated. DNA stained with DAPI, Scale bar = 50 μm.

22. ~Day 40 or later coat 6.5 mm Transwell inserts with hES-qualified Matrigel.
23. Plate 150,000-200,000 iBCs sorted at step 13 or 14 from either ~Day 40, expansion, or cryopreservation, on the apical chamber of an insert in basal cell medium and add 500–600 μL of basal cells medium to the basolateral chamber. Place the culture plate in a humidified incubator at 37°C, 5% CO_2. Feed both top and bottom chambers with basal cell medium every other day.
24. After 4–7 days, confirm confluent or near confluent (>80%) cell monolayer and then change the medium to PneumaCult ALI in both apical and basolateral chambers.
25. The following day, remove the medium from only top chamber and keep feeding every other day from bottom chamber for 2–3 weeks before using.

Note: After one week in ALI, beating cilia should start to be visible under the light microscope. Evaluating specialized cell types such as ciliated cells and secretory cells is recommended to confirm the successful differentiation as needed (Figure 4B).

Note: As few as 30,000 iBCs can be plated in step 23, however, a longer duration of submerged culture may be required.

Note: Other commercially available or non-proprietary defined media (e.g., bronchial epithelial growth medium, BEGM) may also be used in this step but have not been well characterized in this platform.

EXPECTED OUTCOMES

hPSC lines vary in differentiation efficiency and the yield of iBCs. However, starting from two million BU3-NGPT hPSCs in one well of 6 well plate or 0.75 million of non-reporter PSCs in one well of 12 well plate, we obtain roughly two to four million iBCs at Day 40 to 50 time point, resulting in 12 to 24 inserts to establish ALI at the density of 150,000 iBCs per insert. Furthermore, from ~Day40, the iBCs can be expanded with a doubling time of approximately 1.1 days.

Here we breakdown the expected outcomes in each directed differentiation stages. First, we expect >90% cells specify to definitive endoderm quantified by co-expression of CXCR4 and c-KIT surface markers. After differentiating to the NKX2-1^{+} lung progenitor cells, at Day 14–16 of differentiation, 5–25% of NKX2-1^{GFP+} from BU3-NGPT or 10–60% of NKX2-1^{+} from non-reporter PSCs are expected and these lung progenitors are enriched greater than 90% by GFP or cell surface marker-based cell sorting. The retention of lung specification (NKX2-1 positivity) in the sorted NKX2-1^{GFP+} or NKX2-1^{+} cells population can vary from 30% to >90% when assessed at ~Day30 in FGF2+10+DCI+Y medium where about 50% of NKX2-1^{+} cells are TP63^{+} (Figure 2C). When NKX2-1^{GFP+} and TP63$^{tdTomato+}$ cells are sorted in BU3-NGPT at ~Day30, then at Day40+ ~70% of these cells are iBCs, whereas, in non-reporter hPSC differentiation, we obtain 10–60% of cells as NGFR^{+} (and EpCAM^{+}) iBCs, depending on the frequency of NKX2-1^{+} cells that co-express TP63 in ~Day30 culture (Figures 3B and 3C).

LIMITATIONS

Airway epithelium obtained from iBCs in this protocol contains major airway epithelial cells including ciliated, secretory and basal cells. In contrast, rare cell types such as Ionocyte, tuft and pulmonary neuroendocrine cells have not been routinely identified. Therefore, the current protocol may not be suitable for the research requiring the presence of these cell types.

Some iPSC lines may lose NKX2-1 expression significantly during the generation of airway epithelial organoids, typically assessed at ~Day 30. This is likely due to plasticity within a subset of NKX2-1^{+} cells sorted on ~ Day 15 (Hurley et al., 2020). Due to the lack of known cell surface marker(s) to enrich NKX2-1^{+}TP63^{+} airway progenitor cells in this protocol, when a low percentage of NKX2-1^{+} cells (<30%) is identified in cultures from non-reporter iPSCs at the airway organoid stage of the protocol, we do not recommend continuing with the protocol as non-lung endodermal cells may dominate the cultures and sorting for NGFR^{+} cells at later time-points may not be sufficient to generate high quality iBCs.

TROUBLESHOOTING

Problem 1

Poor endoderm specification could lead to unwanted cell linage contamination over the entire period of differentiation, particularly neuronal cells during differentiation of NKX2-1^{+} lung progenitors and airway epithelial organoids (step 4 in "Differentiation to definitive endoderm").

Potential solution

If less than 80% of cells are c-KIT^{+}/CXCR4^{+}, even after testing the range from 54 h through 72 h, consider abandoning the experiment and redo from beginning. Confirm hPSC status, such as pluripotency, described in (Before You Begin).

Problem 2

On day ~15, NKX2-1^{+} lung progenitors (EpCAM^{+}) typically adhere more strongly to the culture plate than other cells (generally, EpCAM^{-}). Insufficient dissociation may leave NKX2-1^{+} lung progenitors behind and incorrectly appear reflect inefficient differentiation into NKX2-1^{+} lung progenitors (low percentage of NKX2-1^{+} lung progenitors) (step 8e in "Differentiation of NKX2-1^{+} lung progenitors into airway epithelial organoids")

Potential solution

Do not shorten the total time, 15–20 min, of Accutase treatment at steps 8c and 8d. If there are still many cells remaining on the culture plate after all detached cells are collected at step 8e, add another mL/well of Accutase, incubate an additional 5 min at 37°C, and recover detached cells.

Problem 3

Due to the lack of sorting strategies to identify NKX2-1^{+}TP63^{+} cells via surface markers at ~Day 30 of non-reporter PSC differentiation, non-lung cell populations may dominate iBCs at later time points if the cultures have a low percentage of NKX2-1^{+} cells (step 8 in "Generation of iPSC-derived basal cells (iBCs)").

Potential solution

When the percentage of NKX2-1^{+} cell population is significantly low (30% or less) during non-reporter PSC differentiation, consider abandoning the experiment and redo from beginning.

Problem 4

In most experiments employing BU3-NGPT or even non-reporter PSCs, iBC cultures from Day40 onward contain little to no non-epithelial cell types (Figures 3B and 3C). However, when differentiating non-reporter PSCs, a small population of NGFR^{+}EpCAM^{-} cells is occasionally detected (step 14d in "Evaluation, isolation and expansion of iBCs").

Potential solution

Include staining of EpCAM and sort on EpCAM^{+} cells.

Problem 5

In non-reporter hPSC differentiation, some PSC lines may yield NGFR^{+} iBCs at extremely low efficiency (< 5%) at Day40 and later. This may also occur in occasional experiments with otherwise well-behaved iPSC lines. This results in a contamination of non-airway cells in downstream applications, such as ALI culture (step 14d in "Evaluation, isolation and expansion of iBCs").

Potential solution

Repeat expansion and sorting to enrich the iBC population, or consider abandoning the experiment.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Brian R. Davis (brian.r.davis@uth.tmc.edu).

Materials availability

Human normal donor iPSC line targeted with NKX2-1GFP and P63tdTomato (BU3-NGPT) is available from the CReM Biobank at Boston University and Boston Medical Center and can be found at http://www.bu.edu/dbin/stemcells/.

Data and code availability

The accession number for the data of BU3-NGPT iBCs or its ALI is deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE142246 as well on the Kotton Lab's Bioinformatics Portal at http://www.bumc.bu.edu/kottonlab/bioinformatics-portal-kotton-lab/.

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AUTHOR CONTRIBUTIONS

S.S., F.J.H., D.N.K., and B.R.D. developed the iBC differentiation protocol and wrote the manuscript. S.S., F.J.H., C.B., and M.L.B. performed differentiation experiments. C.B. and M.L.B. revised and proof-read the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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