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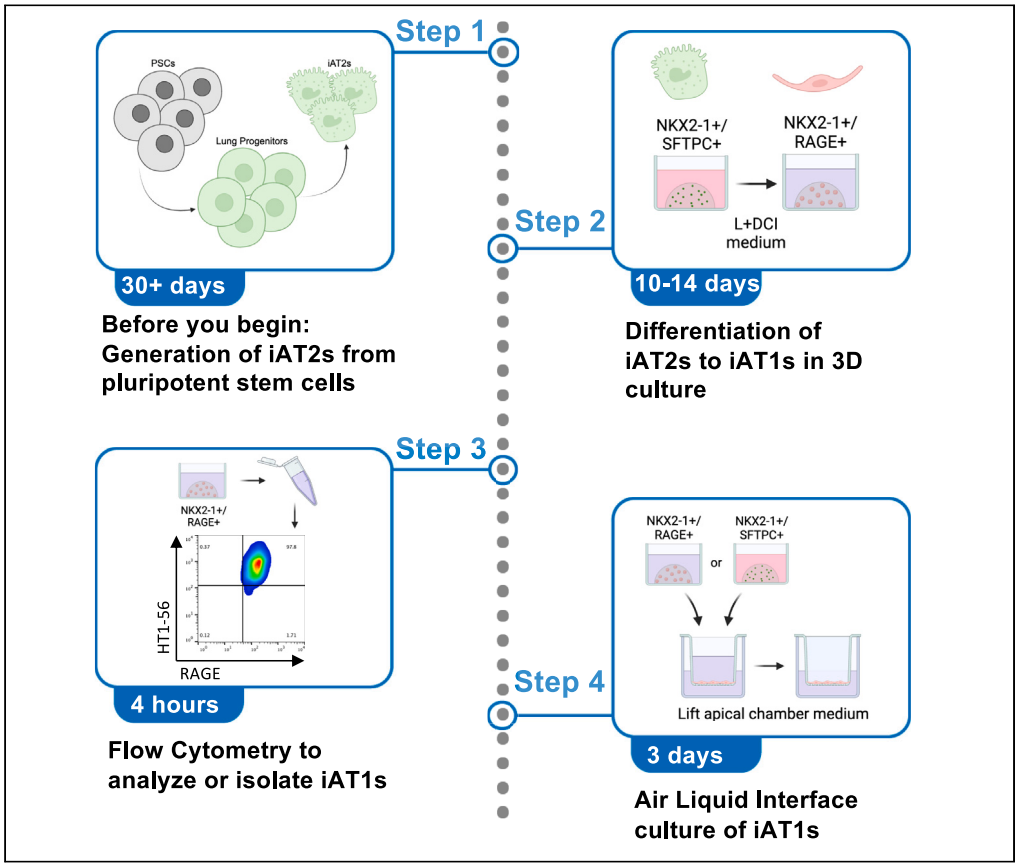
Protocol for the differentiation of human alveolar epithelial type I cells from pluripotent stem cell-derived type II-like cells



Pulmonary alveolar epithelial type I (AT1) cells have a flattened morphology to permit the diffusion of oxygen into the capillaries and historically have been difficult to isolate or maintain in culture. Here, we present a protocol for generating human alveolar type I-like cells (induced pluripotent stem cell-derived AT1s [iAT1s]) from induced pluripotent stem cell-derived alveolar epithelial type II cells (iAT2s) *in vitro*. We describe steps to plate iAT1s in either 3D or air-liquid interface cultures and to analyze or isolate iAT1s via flow cytometry.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Steps for differentiating AT1-like cells from iPSC-derived AT2 cells in 3D culture

Instructions for analyzing AT1-like cells using flow cytometry

Guidance on transitioning AT1-like cells to an air-liquid interface culture

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Protocol for the differentiation of human alveolar epithelial type I cells from pluripotent stem cell-derived type II-like cells

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https://doi.org/10.1016/j.xpro.2025.103667

SUMMARY

Pulmonary alveolar epithelial type I (AT1) cells have a flattened morphology to permit the diffusion of oxygen into the capillaries and historically have been difficult to isolate or maintain in culture. Here, we present a protocol for generating human alveolar type I-like cells (induced pluripotent stem cell-derived AT1s [iAT1s]) from induced pluripotent stem cell-derived alveolar epithelial type II cells (iAT2s) *in vitro*. We describe steps to plate iAT1s in either 3D or air-liquid interface cultures and to analyze or isolate iAT1s via flow cytometry.

For complete details on the use and execution of this protocol, please refer to Burgess et al.[1]

BEFORE YOU BEGIN

The use of human pluripotent stem cells (PSCs), including induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs), may require prior approvals.

The protocol below describes the steps needed to generate cells that recapitulate the molecular and functional characteristics of human alveolar epithelial type I (AT1) cells. We refer to the resulting cells as pluripotent stem cell-derived alveolar epithelial type I cells (iAT1s) and detail here our approach for deriving these cells by first generating human PSC-derived alveolar epithelial type II cells (iAT2s) followed by their differentiation into iAT1s using a defined, serum-free medium. Our medium drives a transcriptomic shift from the iAT2 program to a type I-like program, and the text below details methods for quantitation, purification, and characterization of the resulting iAT1s by flow cytometry as well as approaches for plating iAT1s in air-liquid interface (ALI) cultures. Before beginning this protocol, first generate iAT2s from iPSCs using the 30+ day protocol described in Jacob et al.[2]

This protocol was optimized using the BU3 NGAT iPSC line,[1] which is a bifluorescent reporter line carrying NKX2-1^{GFP} (NG) and AGER^{tdTomato}(AT). However, we have also used this protocol to generate iAT1s from multiple other iPSC lines lacking AT1 reporters as well as embryonic stem cell lines. We have outlined methods here to track and isolate iAT1s in the absence of an AT1 reporter via sorting cells that have been immunostained to identify AT1-specific cell-surface proteins.

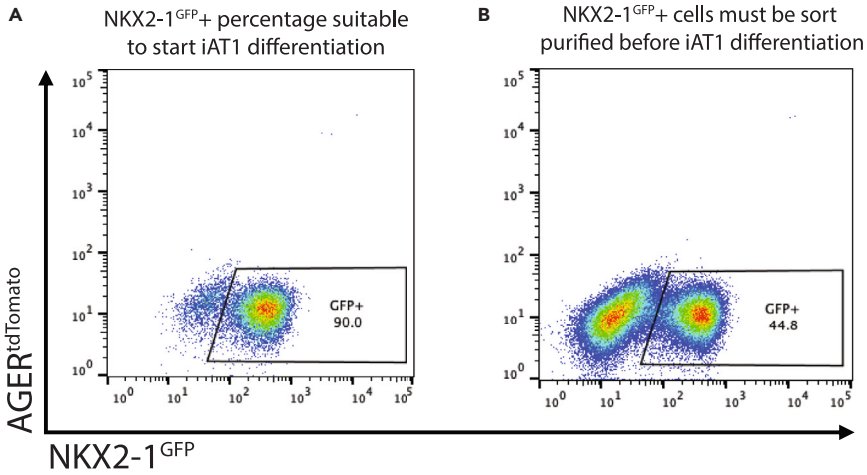

Figure 1. Flow cytometry of iAT2s with NKX2-1^{GFP} and AGER^{tdTomato} reporters (BU3 NGAT)

(A) iAT2s are 90% NKX2-1^{GFP}+ and may be plated to begin iAT1 differentiation.

(B) The NKX2-1^{GFP}+ population in this culture has dropped well below 80% and should be sort purified for GFP+ cells before beginning iAT1 differentiation.

Generation of iAT2s from iPSCs

Timing: 30+ days

1. Generate iAT2s from iPSCs using the protocol described in Jacob et al.[2]
2. Maintain iAT2s in 3D culture as described in Jacob et al. for up to one year, until ready to begin iAT1 differentiation.

Note: iAT2s occasionally need to be sorted to maintain a pure population of type 2-like cells. As iAT1s are differentiated from iAT2s, ensure that the starting iAT2 culture is >80% NKX2-1+ before beginning the iAT1 differentiation protocol (Figure 1). If the iAT2 population is <80% NKX2-1+, sort purify cells before beginning the iAT1 differentiation protocol to ensure a high differentiation efficiency. For cell lines without a NKX2-1 reporter, sorting for CPM^{high} cells will generate a relatively pure population of distal alveolar NKX2-1+ cells[2,3] or sorting for SLC34A2[4,5] will purify more mature SFTPC+ cells.

Preparation of Transwell inserts for iAT1 ALI culture

Timing: 30 min

3. Dilute hESC qualified Matrigel.
   a. Thaw a 5 mL vial of Matrigel on ice.
   b. Keeping tubes on ice, divide Matrigel into 1.5 mL microcentrifuge tubes according to the dilution factor provided on the Corning website for each individual lot number and store.
   c. Resuspend one aliquot of Matrigel in DMEM/F12 according to the manufacturer's instructions.
4. Coat 6.5 mm Transwell inserts with hESC qualified Matrigel.
   a. Add 100 μL of diluted Matrigel to each 6.5 mm Transwell insert.
   b. Place the plate containing coated Transwell inserts in a 37°C incubator for at least 10 min.

Note: Transwells can be coated in advance and stored at 4°C for up to two weeks. Seal plate with Parafilm and store flat. Place plate in 37°C incubator for at least 10 min before plating ALI cultures.

Note: Aliquots of hESC qualified Matrigel may be stored at −80°C until the expiration date specified by the manufacturer, according to each individual lot number. Aliquots may be thawed on ice before use as needed.

Institutional permissions

All iPSC differentiations were performed under regulatory approval of the Institutional Review Board of Boston University (protocol H33122).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat anti-RAGE antibody (1:100)	R&D Systems	Cat#AF1145
Alexa Fluor 647 donkey anti-goat antibody (1:500)	Invitrogen	Cat#A21447
Mouse anti-SLC34A2 antibody (MX35) (1:200)	Gift from Dr. Gerd Ritter, Memorial Sloan Kettering[6]	MX35
Mouse anti-carboxypeptidase M (CPM) antibody(1:200)	Wako	Cat# 014-27501
Chemicals, peptides, and recombinant proteins		
1-Thioglycerol (MTG)	Sigma	Cat#M6145
Ascorbic acid	Sigma	Cat#A4544
Y-27632 (ROCK inhibitor)	Tocris	Cat#1254
Dispase	Gibco	Cat#17105-041
Calcein blue	Thermo Fisher Scientific	Cat#C1429
CHIR99021	Tocris	Cat#4423
rhKGF (FGF7)	Fisher Scientific	Cat#251KG050
TRULI (LATS-IN-1)	MedChem Express	Cat#HY-138489
Dexamethasone	Sigma	Cat#D4902
8-Bromoadenosine 3′, 5′-cyclic monophosphate sodium salt (cAMP)	Sigma	Cat#B7880
3-Isobutyl-1-methylxanthine (IBMX)	Sigma	Cat#I5879
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat#D2650
Deposited data		
scRNA-seq data: 3D iAT1s vs. ALI iAT1s	Burgess et al.[1]	GEO: GSE221344
Experimental models: Cell lines		
Human: normal donor iPSC line targeted with NKX2-1^{GFP} and AGER^{tdTomato} (BU3 NGAT)	Kotton Lab; Burgess et al.[1]	http://stemcellbank.bu.edu
Human: donor iPSC line targeted with SFTPC^{tdTomato} (SPC2-ST-B2)	Kotton Lab; Hurley et al.[7]; Alysandratos et al.[8]	http://stemcellbank.bu.edu
Software and algorithms		
Prism	GraphPad	https://www.graphpad.com
FlowJo	Becton Dickinson & Company	https://flowjo.com/solutions/flowjo
Fiji (v.2.14.0/1.54f)	Schindelin et al.[9]	https://imagej.net/software/fiji/
Other		
Growth factor reduced 3D Matrigel	Corning	Cat#356231
hESC-qualified Matrigel	Corning	Cat#354277
IMDM	Gibco	Cat#12440053
Glutamax	Gibco	Cat#35050-061
Ham's F12	Cellgro	Cat#10-080-CV
B27 supplement	Gibco	Cat#17504-44
N2 supplement	Gibco	Cat#17502-048
Primocin	Invitrogen	Cat#NC9141851
7.5% BSA Fraction V	Gibco	Cat#15260-0037
0.05% Trypsin-EDTA	Gibco	Cat#25-300-062
Characterized fetal bovine serum (FBS)	Cytiva	Cat#SH30071.03

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dulbecco's PBS (no calcium, no magnesium)	Gibco	Cat#14190144
Hank's buffered saline solution (HBSS, no calcium, no magnesium, no phenol red)	Gibco	Cat#14175095
Trypan blue	Thermo Fisher Scientific	Cat#1520061
500 mL disposable sterile filter systems	Millipore	Cat#09-761-107
SteriFlip sterile disposable vacuum filter unit	EMD Millipore	Cat# SCGP00525
6.5 mm Transwell with 0.4 μm pore polyester membrane insert	Corning	Cat#3470
Falcon 12 well culture plate	Corning	Cat#353043
1.5 mL microcentrifuge tubes	USA Scientific	Cat#05-402-25
Luna II automated cell counter	Logos Biosystems	Cat#L40001
Cell sorter: MoFlo Astrios	Beckman Coulter	Cat#B52102
Flow cytometer	Stratedigm	Model #S1000EXi
Multipurpose centrifuge	Eppendorf	Model #5810R
Round-bottom polystyrene tubes	Fisher Scientific	Cat#06666C

MATERIALS AND EQUIPMENT

cSFDM (complete serum free differentiation medium)

Reagent	Final concentration	Amount
375 mL IMDM	–	375 mL
125 mL Ham's F12	–	125 mL
B-27 (with RA) supplement	1%	5 mL
N-2 supplement	0.5%	2.5 mL
3.3 mL BSA (7.5% stock)	0.05%	3.3 mL
Primocin (50 mg/mL stock)	100 μg/mL	1 mL
Glutamax 100×	1×	5 mL
Ascorbic Acid (50 mg/mL stock)	50 μg/mL	500 μL
MTG	4.5×10^{-4} M	19.5 μL
Total	–	**517.32 mL**

Filter through a 500 mL disposable filter system and store protected from light at 4°C for up to one month.

10× CI

Reagent	Final concentration	Amount
cSFDM Base	–	50 mL
8-bromoadenosine 3′, 5′-cyclic monophosphate sodium salt (cAMP)	0.1 mM	21.5 mg
IBMX (100 mM)	1 mM	500 uL
Total	**10×**	**50.5 mL**

Filter solution through a SteriFlip and store protected from light at 4°C for up to one month.

CK + DCI Medium

Reagent	Final concentration	Amount
cSFDM Base	–	45 mL
10× CI	1×	5 mL
CHIR99021 (3 mM stock)	3 uM	50 μL
rhKGF (10 μg/mL stock)	10 ng/mL	50 μL
Dexamethasone (100 μM stock)	50 nM	25 μL
Total	-	**50.125 mL**

Store protected from light at 4°C for up to one month.

L + DCI Medium		
Reagent	Final concentration	Amount
cSFDM Base	–	45 mL
10× CI	1×	5 mL
LATS-IN-1 (10 mM stock)	10 μM	50 μL
Dexamethasone (100 μM stock)	50 nM	25 μL
Total	–	**50.075 mL**
Store protected from light at 4°C for up to one month.		

Flow cytometry sorting buffer (FACS buffer)		
Reagent	Final concentration	Amount
HBSS	–	49 mL
Characterized FBS	2%	1 mL
Primocin	100 μg/mL	100 μL
Total	–	50.1 mL
Filter solution through a SteriFlip and store at 4°C for up to one month.		

STEP-BY-STEP METHOD DETAILS

iAT1 differentiation from iAT2s in 3D culture

Timing: 10–14 days

In this step, we provide details of how to generate iAT1s from iAT2s in 3D culture.

1. Passage iAT2s as single cells into 3D Matrigel droplets (2 h).
   a. Aspirate medium from the well.

   ⚠ CRITICAL: Take care not to aspirate Matrigel droplet.

   b. Add 1 mL of Dispase (2 mg/mL) per well and incubate at 37°C for 1 h.
      i. Pipette up and down 1-2 times at 30 min to help break apart Matrigel drop.
   c. Transfer all Dispase and dissociated organoids into a 15 mL conical.
      i. Rinse the well with DMEM or empty medium to collect all organoids.
      ii. Add 10 mL DMEM or empty medium to the 15 mL conical to wash organoids.
   d. Centrifuge at 300 g for 5 min and aspirate supernatant. Leave as little supernatant as possible.
   e. Resuspend cells in 1 mL 0.05% trypsin per well and incubate at 37°C for 10–12 min, until pipetting 3-5 times results in a single cell suspension.

   Note: Cells are sensitive to overpipetting.

   Note: To visualize cells under a microscope during dissociation, transfer trypsin + cells to a tissue culture plate.

   f. Add 1 mL of FBS per 1 mL trypsin to stop dissociation. Add 10 mL DMEM to wash the cells and spin at 300 g for 5 min.
   g. Count cells and resuspend in 3D Matrigel at 400 cells/μL Matrigel. Plate one 50–100 μL Matrigel droplet per well in a 12-well plate, avoiding bubbles.

   Note: Because iAT1s must be generated fresh from iAT2s each passage, we recommend plating at least 2 extra wells of iAT2s at this step that will be maintained in CK + DCI, to keep a stock of iAT2s.

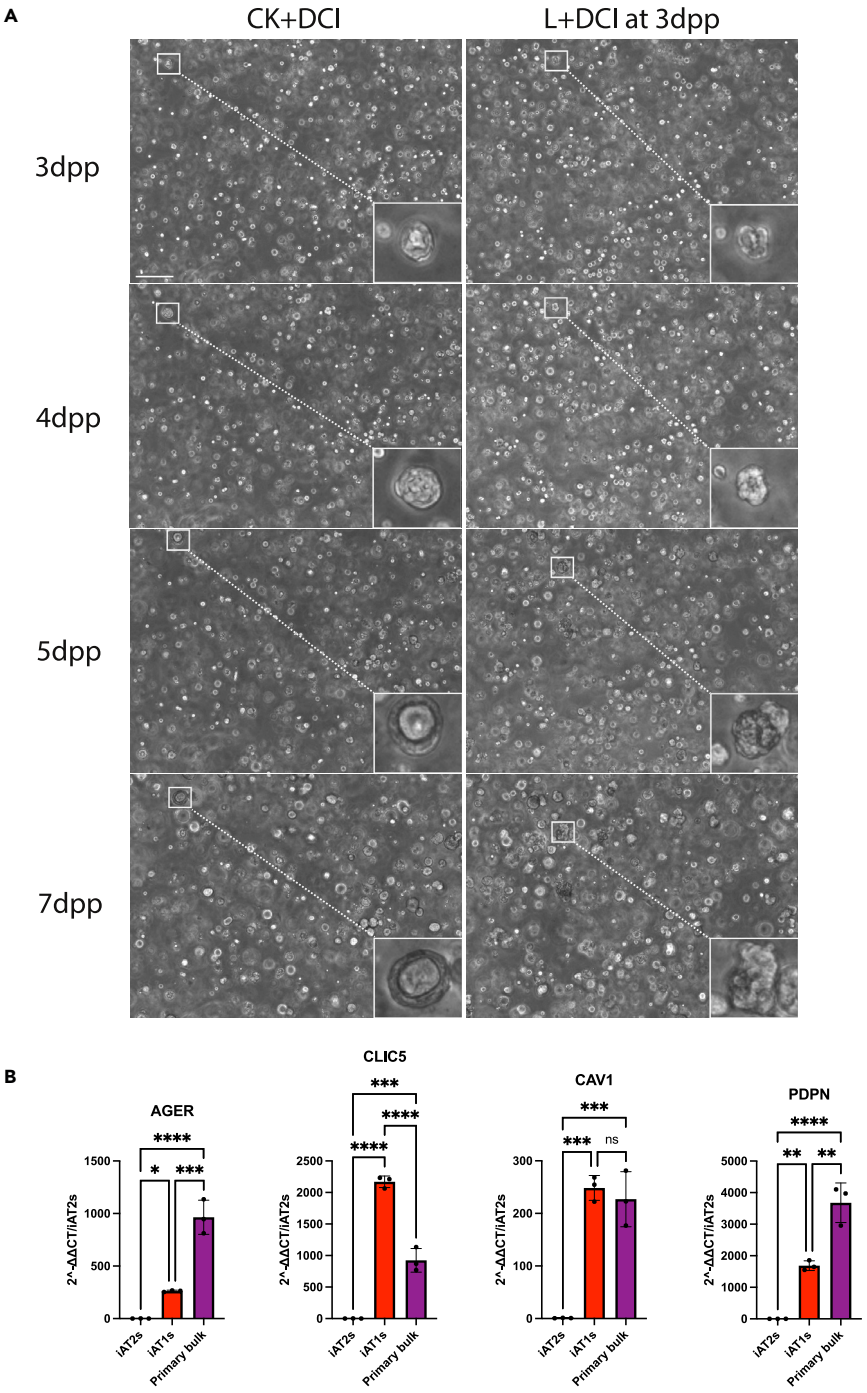

Figure 2. iAT1s undergo morphological and transcriptomic changes after transitioning to L + DCI

(A) Representative morphology of BU3 NGAT iAT2s and iAT1s at 3, 4, 5, and 7 days post passage (dpp). iAT1s are switched to L + DCI medium at 3 dpp, and iAT2s are kept in CK + DCI medium. Scale bar = 200 μm.

(B) RT-qPCR for expression of AT1 marker genes in SPC2-ST-B2 iAT2s and iAT1s (13 dpp), and primary bulk adult human distal lung. Expression is normalized to an 18S control and fold change is calculated by $2^{-\Delta\Delta Ct}$, relative to iAT2s. (N = 3, one-way Analysis of Variance (ANOVA) with Tukey multiple comparisons).

*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001; bars represent mean ± SD for all panels.

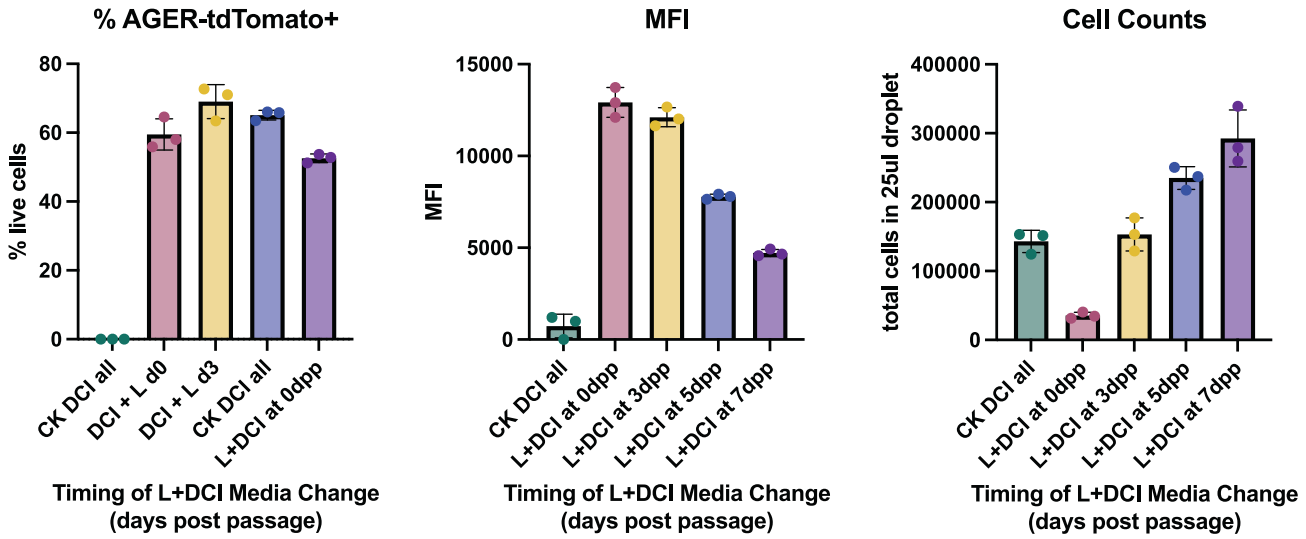

Figure 3. Differentiating cells to iAT1s 3 days post passage (dpp) maximizes tdTom+ mean fluorescence intensity (MFI) in BU3 NGATs

Percent AGER^{tdTomato}+ cells, MFI, and cell counts of BU3 NGAT iAT1s after switching the medium to L + DCI 0, 3, 5, or 7 dpp. iAT1s were collected at 14 dpp. Bars represent mean ± SD for all panels.

h. Place in 37°C incubator for 15–20 min to allow Matrigel to solidify.
   i. Do not add medium before Matrigel has solidified.
i. Add 1 mL CK+DCI+ 10 μM Y-27632 over Matrigel drop. Pipette medium onto side of well to avoid disrupting Matrigel droplet.

2. After 72 h in CK+DCI+Y, change medium to L + DCI (10 min/day hands on, 7–11 days).
   a. Aspirate all CK+DCI+Y medium from the Matrigel drop.
   b. Add 1 mL L + DCI per well.
   c. Refeed every 48 h with L + DCI.

⚠ CRITICAL: iAT1s will proliferate for the first few days post medium change but will then decrease proliferation and will not proliferate upon passaging. iAT1s must be generated fresh from iAT2s each passage.

Note: Cells will begin to turn on AT1 genes within 24–72 h post medium change (Figure 2; see also our published RNA-sequencing time series[1]).

Note: AT1 marker gene expression will continue to increase over two weeks in culture.

Note: Medium can be changed from CK + DCI at a time point other than day 3 post passage, but this will affect final cell number and AT1 marker expression. Day 3 was chosen based on optimization using the BU3NGAT iPSC line to maximize TdTomato mean fluorescence intensity (MFI) at 14 days post passage (Figure 3).

iAT1 quantification or isolation via flow cytometry

Timing: 4 h

In this step, we provide details of how to prepare iAT1s for antibody-based fluorescence activated cell sorting (FACS) using the AT1-specific cell surface marker RAGE, which may be performed to assess the efficiency of iAT1 differentiation or to isolate iAT1s in the absence of an AGER^{tdTomato} fluorescent reporter.

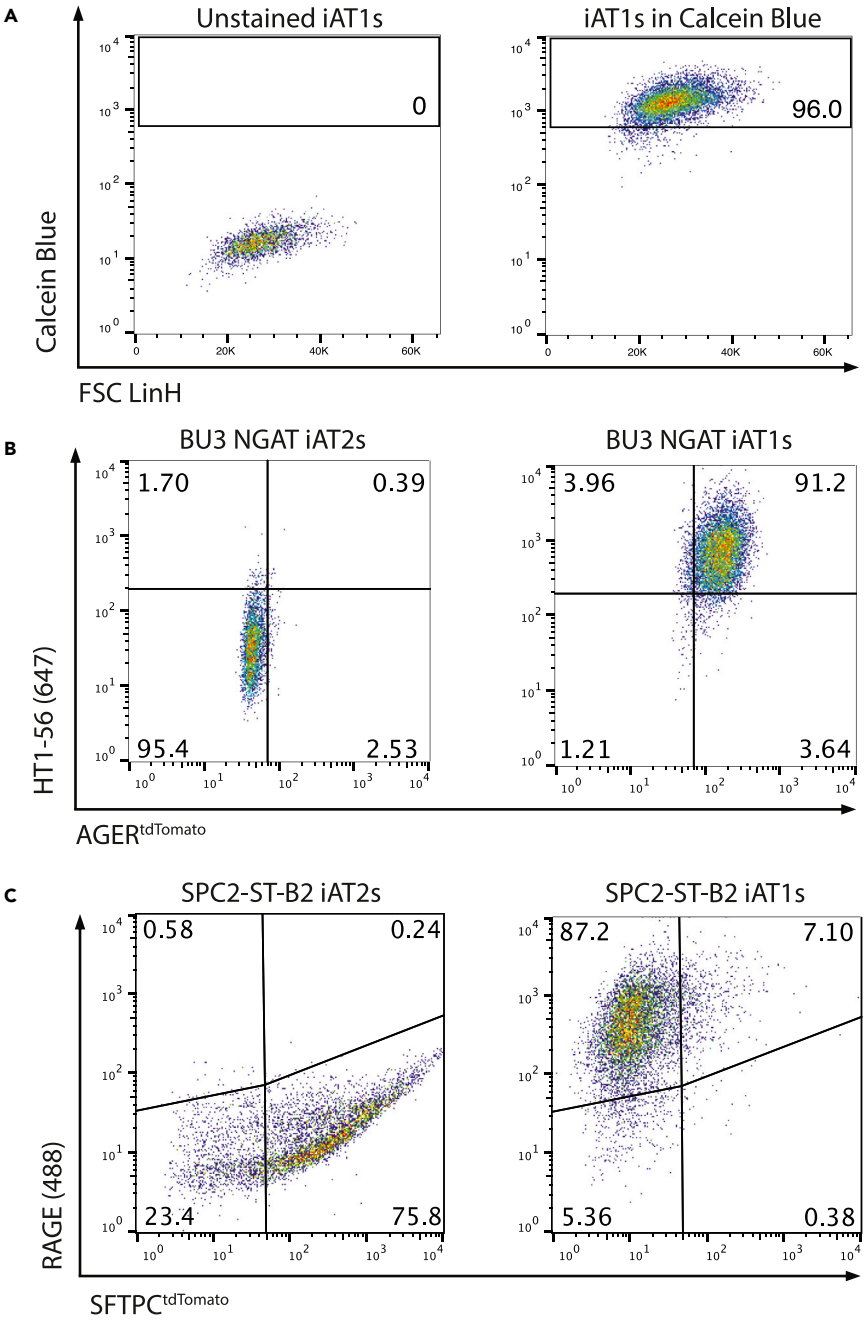

Figure 4. Representative flow cytometry plots of HTI-56 and RAGE staining of iAT2s and iAT1s

(A) Viability of iAT1s following single cell dissociation, as described in steps 3a-3f.

(B) Flow cytometry of HTI-56-stained BU3 NGAT iAT1s and iAT2s at 10 days post passage.

(C) Flow cytometry of RAGE-stained SPC2-ST-B2 iAT1s and iAT2s at 10 days post passage.

Note: We recommend preparing a parallel staining of an iAT2 sample with the same primary + secondary antibody staining protocol used to sort the iAT1 stained sample. The stained iAT2s can then be used to set gates, gating all iAT2s as negative for RAGE (or HT1-56, see note below). After dissociating iAT2s to single cells (steps 1a-f), the listed protocol in steps 3-6 may be used to stain iAT2s (Figure 4).

Note: The goat anti-RAGE primary antibody we recommend (R&D systems, Cat#AF1145) is a polyclonal antibody, which is then detected using a fluorescence-conjugated secondary antibody, as detailed below. Since FACS gating controls for polyclonal primary antibodies are problematic (isotype control antibodies are only relevant for monoclonal antibody staining), we recommend preparing a parallel "secondary antibody only" control sample that is not incubated in primary antibody, to determine the increase in signal due to nonspecific binding of the secondary antibody.

Note: HT1-56 (Terrace Biotech TB-29AHT1-56) monoclonal antibody immunostaining may also be used at the same dilution as the anti-RAGE antibody to quantify or sort iAT1 cells (1:100).[1] In other words, the number of cells, dilution factors, and volumes listed here for RAGE immunostaining are suitable for HT1-56 as well. The manufacturer does not produce a mouse IgG1 isotype control for this antibody, and so the "secondary only" control should also be used when staining for HT1-56. We recommend Alexa Fluor 647 donkey anti-mouse (Invitrogen Cat#A31571) as the secondary antibody for HT1-56 staining (1:500). At the time of publication, the antigen bound by the HT1-56 antibody has not yet been determined.

3. Dissociate iAT1s into single cells.
   a. Aspirate L + DCI from Matrigel droplet. Do not disrupt the Matrigel droplet.
   b. Add 1 mL Dispase (2 mg/mL) to the well to break up the Matrigel droplet. Incubate at 37°C for 1 h
      i. Pipette up and down 1-2 times at 30 min to help break apart Matrigel droplet.
   c. Transfer 1 mL of iAT1s in Dispase to a 15 mL conical and add 10 mL PBS or empty medium to wash.
   d. Centrifuge 300 g for 5 min and aspirate supernatant.
   e. Resuspend in 1 mL 0.05% Trypsin per well and incubate at 37°C for up to 15 min, pipetting 3-5 times after 7 min.
   f. Stop digestion with 1 mL FBS per mL of Trypsin, add 10 mL of PBS or empty medium to wash, and spin at 300 g for 5 min.
   g. Resuspend cells in 1 mL flow cytometry sorting (FACS) buffer.
   h. Count cells.
4. Primary antibody incubation
   a. Aliquot 1 × 10^5 cells each into three 1.5 mL mini centrifuge tubes. These three aliquots will be the "stained" sample, and the "secondary only" and "unstained" controls.

   Note: If staining iAT1s and iAT2s at the same time, three aliquots of 1 × 10^5 cells/100 μL each should be generated for each cell type.

   b. Centrifuge cells at 300 × g for 5 min.
   c. Aspirate supernatant from each cell pellet.
   d. Resuspend each cell pellet in 100 μL of FACS buffer per 1 × 10^5 cells.
   e. Add 1 μL of primary antibody (goat anti-RAGE, R&D systems, Cat#AF1145) to the "stained" sample (1:100).

   Note: We stain using 1 μL of primary antibody per 1 × 10^5 cells in a staining volume of 100 μL, and these numbers should be adjusted for larger or smaller cell numbers while keeping all ratios of antibody to cell number to staining volume consistent. Hence, if 1 × 10^6 cells are to be stained we use 10 μL of antibody diluted in a 1 mL staining volume.

   f. Incubate all samples for 45 min on ice.
   g. Add 1 mL FACS buffer to each sample to wash cells and centrifuge all samples at 300 × g for 5 min.
5. Secondary antibody incubation

a. Add 1 μL of secondary antibody \ (Alexa Fluor 647 donkey anti-goat, Invitrogen Cat#A21447) to 500 μL of FACS buffer to dilute (1:500).

Note: You will only need 100 μL of diluted secondary antibody per 1 × 10^5 cell sample. However, we recommend making a minimum of 500 μL diluted antibody with which to resuspend cells to eliminate the need to pipette volumes under 1 μL, which may compromise pipetting accuracy. If you require larger volumes of diluted secondary, adjust volumes so that the secondary antibody is added to the FACS buffer at a ratio of 1:500.

Note: Alternative colors or host species may be used for the secondary antibody for RAGE immunostaining, provided the secondary antibody is anti-goat.

Note: For HT1-56 staining, we recommend Alexa Fluor 647 donkey anti-mouse (Invitrogen Cat#A31571).

b. Resuspend each of the "stained" and "secondary only" samples (1 × 10^5 cells in each) in 100 μL of secondary antibody staining buffer.
c. Resuspend the "unstained" sample in 100 μL FACS buffer.
d. Incubate all samples on ice and protected from light for 20 min.
e. Add 1 mL FACS buffer to each sample to wash cells and centrifuge all samples at 300 × g for 5 min.

6. Prepare samples for flow cytometry.
   a. Make a 1000× (10 mM) stock solution of Calcein blue by reconstituting according to the listed molecular weight in the appropriate volume of DMSO.

   Note: 10-μL aliquots of 1000× Calcein blue stock may be stored at −20°C for up to 1 year.

   b. Add 1 μL of Calcein blue to 1 mL FACS buffer for a final concentration of 10 nM (FACS + Calcein blue buffer).
   c. If sorting cells, add Y-27632 to the FACS + Calcein blue buffer for a final concentration of 10 nM (FACS + Calcein blue + Y buffer).

   Note: Sorting is stressful on the cells, and the addition of Y-27632 to the FACS buffer improves viability of the collected cells. If the cells are only being analyzed by flow cytometry, adding Y-27632 is not required.

   d. Resuspend each sample in 300 μL FACS + Calcein blue (+ Y) buffer.
   e. Filter each sample through a 40 μm cell strainer to ensure single-cell suspension. Use a new strainer for each sample to avoid cross-contamination of the stained sample and controls.
   f. Transfer samples to a round-bottom polystyrene tube (or polypropylene tube, depending on flow cytometer's specifications).
   g. Keep on ice and protected from light until ready to analyze.
7. Analyze or isolate cells with flow cytometry.

Note: As described above, stained iAT2s can be used to set gates. For HT1-56 immunostaining, use HT1-56-stained iAT2s, gating the iAT2 population as HT1-$56^{negative}$. Although HT1-56 stained iAT2s will show a slight increase in fluorescent signal over unstained or secondary-only iAT2s, gating on stained iAT2s will still enable clear delineation between iAT1s and iAT2s (Figure 4B).

ALI culture of iAT1s

Timing: 3 days (2 h hands-on)

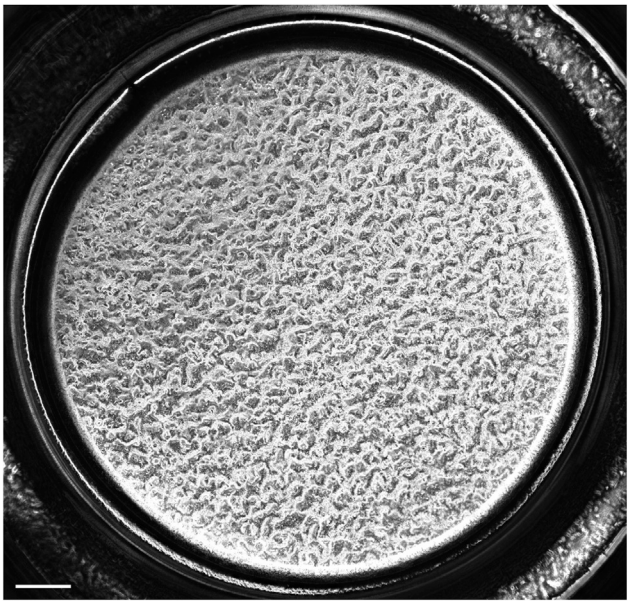

Figure 5. Phase contrast image of a live iAT1 ALI culture
Cells are imaged 14 days after plating (11 days after airlifting medium), plated at 100 k cells/well on a 6.5 mm Transwell. Scale bar = 500 μm.

In this step, iAT1s are transitioned from 3D culture onto Transwell inserts to establish an air liquid interface (ALI) culture (Figure 5; also known as iAT1 3D differentiation prior to ALI plating after "P1" passaging).

Note: We recommend growing iAT1s in 3D for 6–12 days post medium change to L + DCI before dissociating to single cell suspension for transition to ALI culture.

Note: Coat Transwell inserts with Matrigel before beginning this step (see "before you begin: Preparation of Transwell inserts for iAT1 ALI culture").

8. After generating iAT1s in 3D from iAT2s (steps 1-2), dissociate iAT1s into a single cell suspension, as described in step 3 [See step 3a–3f.].
9. Dilute iAT1s to the appropriate concentration for plating ALI cultures.
   a. Spin single cell suspension at 300 g for 5 min.
   b. Aspirate supernatant and resuspend pellet in 1 mL L+DCI+Y-27632.
   c. Count cells and dilute as needed to plate 50–200 k cells in 100 μL L+DCI+Y-27632 medium per Transwell.

Note: Dissociation as described in steps 3a–3f has been optimized for viability of iAT1 cells, and the majority of cells should be viable after dissociation (Figure 4A). Confirm cell viability with Trypan Blue or flow cytometry before proceeding.

Note: Cells may be spun at 300 g for 5 min and resuspended in a lower volume of L+DCI+Y-27632 if cell suspension is too dilute.

Note: Cells can be plated at 50 k-200 k cells per 6.5 mm insert. Lower density plating leads to increased surface area per cell as iAT1s spread to cover available surface area.

10. Plate iAT1s on Transwell inserts.

a. Aspirate excess liquid from Matrigel-coated Transwell inserts and rinse inserts with 100 μL PBS. Aspirate PBS and add 100 μL of L+DCI+Y-27632 to the apical chamber of each transwell.

Note: Work quickly after aspirating liquid from Transwell insert to prevent Matrigel coating from drying out.

b. Add 100 μL of iAT1s at the desired density to the apical chamber of each Transwell insert.
c. Gently rock plate in a cross pattern to evenly distribute cells across insert.
d. Add 500 μL L+DCI+Y-27632 to the basal chamber of the Transwell.

11. After 72 h, aspirate medium from the apical chamber. Refeed the basal chamber with L + DCI.

Note: iAT1s at ALI culture should maintain an epithelial barrier. After aspiration of the apical medium, there should be no leakage from the basal chamber to the apical side of the transwell (see Troubleshooting, Problem 3).

12. Refeed basal chamber with L + DCI every 48 h.

Note: The protocol listed here refers to the "P1" ALI protocol described in Burgess et al.[1] iAT1 ALI cultures may also be plated using the "P0" protocol, where 3D iAT2s are dissociated instead of 3D iAT1s. See step 14 for the P0 protocol.

P0 ALI culture of iAT1s

Timing: 3 days (2 h hands-on)

In this step, iAT1 ALI cultures are generated by transitioning iAT2s from 3D culture in CK + DCI medium onto Transwell inserts in L + DCI medium (also known as iAT1 3D differentiation upon ALI plating, "P0").

Note: Coat Transwell inserts with Matrigel before beginning this step (see "before you begin: preparation of Transwell inserts for iAT1 ALI culture").

13. 7–12 days after passaging, dissociate iAT2s into a single cell suspension, as described in steps 1a-f.
    a. Dilute iAT2s to the appropriate concentration for plating ALI cultures.
14. Spin single cell suspension at 300 g for 5 min.
    a. Aspirate supernatant and resuspend pellet in 1 mL L+DCI+Y-27632.

Note: From this point on, use L + DCI (+Y-27632 when specified) medium. By changing the medium from CK + DCI to L + DCI, iAT2s will differentiate to iAT1s in the days following ALI plating.

b. Count cells and dilute as needed to plate 100 k cells in 100 μL L+DCI+Y-27632 medium per Transwell.

Note: Cells may be spun at 300 g for 5 min and resuspended in a lower volume of L+DCI+Y-27632 if cell suspension is too dilute.

Note: Cells can be plated at 100 k cells per 6.5 mm insert for P0 ALIs. Lower density plating may be successful, but Transwells should be monitored following airlift (step 17) to ensure no leakage of the basal medium to the apical chamber.

15. Plate cells on Transwell inserts.
    a. Aspirate excess liquid from Matrigel-coated Transwell inserts and rinse inserts with 100 μL PBS. Aspirate PBS and add 100 μL of L+DCI+Y-27632 to the apical chamber of each Transwell.

Note: Work quickly after aspirating liquid from Transwell insert to prevent Matrigel coating from drying out.

    b. Add 100 μL of cells to the apical chamber of each Transwell insert.
    c. Gently rock plate in a cross pattern to evenly distribute cells across insert.
    d. Add 500 μL L+DCI+Y-27632 to the basal chamber of the Transwell.
16. After 72 h, aspirate medium from the apical chamber. Refeed the basal chamber with L + DCI.
17. Refeed basal chamber with L + DCI every 48 h.

Note: We recommend performing experiments 7–14 days after plating.

Assessing the epithelial barrier function of iAT1 ALIs with transepithelial electrical resistance

Timing: 30 min

In this step, we describe how to analyze iAT1 ALIs with transepithelial electrical resistance (TEER).

18. Add 200 μL of L + DCI medium to the apical chamber of each ALI.
19. Sterilize the probes of the voltohmmeter by dipping in 70% ethanol, then dip the probes in medium to remove any excess ethanol.
20. To take a reading, place the prongs of the probe so that the shorter probe is submerged in the apical medium, and the longer probe is submerged in the basal chamber medium through one of the openings in the transwell walls. Take readings at 3 locations in each well.

⚠ CRITICAL: Do not touch cell layer with probe.

21. To calculate TEER, we take the difference between the mean sample reading and the mean blank reading, and multiply this value by the area of the transwell (0.33 cm^2 for 6.5 mm inserts).

EXPECTED OUTCOMES

This protocol outlines an efficient method to generate iAT1s from iAT2s. We apply a serum free, feeder-free defined medium to induce iAT2 to iAT1 differentiation. After 24–72 h, L + DCI induces robust morphologic and transcriptomic changes in iAT2s (Figure 2; see also our published RNA-sequencing time series[1]), with upregulation of AT1 marker genes, which continue to increase over the following two weeks. Immunostaining for RAGE or HT1-56 should yield >80% positive cells, as quantified by flow cytometry (Figure 4).

When plated at ALI culture, iAT1s spread to cover available surface area, resulting in thin, flattened cells resembling *in vivo* AT1 morphology.[1] Cells maintain an epithelial barrier, with increasing transepithelial electrical resistance (TEER) over time after plating.[1] iAT1s at ALI culture can maintain this non-leaking barrier for at least 3 months, though we typically use the cells for most experiments within 7–14 days. After transitioning from 3D to ALI cultures, iAT1s upregulate genes associated with ECM components, including the components of laminin-332 (*LAMA3*, *LAMB3*, and *LAMC2*), as well as transcripts encoding the signaling ligands *PDGFA*, *SHH*, *WNT7A*, and *VEGFA*.[1] User-friendly visualizations of complete gene expression in 3D iAT2s, 3D iAT1s, and iAT1s at ALI culture at the single cell level (as published in Burgess et al.[1]) may be viewed at our online bioinformatics portal at www.kottonlab.com or at the individual Shiny apps posted at this portal

as: https://crem-bu.shinyapps.io/scRNAseq_iAT1_3D_ALI_comparison/ with additional time series profiles of iAT2 to iAT1 differentiation: https://crem-bu.shinyapps.io/scRNAseq_iAT1_differentiation_time_series/.

LIMITATIONS

This protocol generates cells that mimic molecular and functional properties of human AT1 cells. However, although the protocol initiates a transcriptomic shift from a type 2-like to a type 1-like program, expression levels of many canonical AT1 marker genes are significantly lower than in primary AT1 cells *in vivo*, suggesting that iAT1s may be immature as compared to their *in vivo* counterparts. Similar issues affect primary cell cultures as primary adult human AT2 cells also express lower levels of AT2 marker genes than their *in vivo* counterparts[10] and when transitioned *in vitro* to primary AT1-like cells also express low levels of AT1 markers.[1] Additionally, to maintain their AT1 phenotype, iAT1s must be kept in L + DCI. Removal of the LATS inhibitor from the medium results in a downregulation of the AT1 transcriptomic program. This may be prohibitive to developing co-cultures of iAT1s with cell types that do not maintain their phenotype when exposed to LATS inhibition.

TROUBLESHOOTING

Problem 1

The efficiency of iAT1 differentiation is low (related to step 7).

Potential solution

Before differentiating iAT2s to iAT1s, ensure that the majority of iAT2s are NKX2-1+. This may be assessed by flow cytometry for NKX2-1^{GFP}, in cell lines with a fluorescent reporter (Figure 1). In the absence of this reporter, surface staining for carboxypeptidase M (CPM) can be used to enrich for distal alveolar NKX2-1+ cells via FACS.[2,3] iAT2 cultures with <80% NKX2-1 ^{GFP}+ or CPM+ cells should be sort purified prior to iAT1 differentiation. In addition, variance in iAT1 differentiation efficiencies across iPSC lines of different genetic backgrounds is likely, but has not yet been carefully characterized from a wide diversity of donors to date.[1] We suggest using a line with established high iAT1 competence from the literature if you are having trouble with iAT1 differentiation efficiency.

Problem 2

iAT1s fail to fully dissociate to single cells after 15 min in Trypsin (related to step 3).

Potential solution

iAT1s take longer to dissociate to a single cell suspension than iAT2s. An additional 1 mL of Trypsin may be added at the halfway point if the Trypsin has become pale. After stopping with FBS, large clumps of cells may be removed from cell suspension by filtering through a 40 μM cell strainer. If necessary, filtering should be performed before counting cells.

Problem 3

iAT1s fail to form an epithelial barrier when plated at ALI culture (related to step 11).

Potential solution

iAT1s form a flattened epithelial barrier even when plated at low densities (50 k cells per 6.5 mm Transwell insert). First, ensure that the efficiency of iAT1 differentiation is >80%. Confirm that iAT1s have fully dissociated to single cell solution before plating the cells at ALI culture by performing Trypsin dissociation (step 3e) in a tissue culture plate, allowing for visualization of cells under a microscope every few minutes. After transferring cells to Transwell (step 10b), ensure that cells are evenly distributed over the Transwell with no obvious clumps of cells. If after Trypsin dissociation there are still large aggregates of cells, the cell suspension of iAT1s may be filtered through a 40 μM cell strainer before plating at ALI.

Problem 4

Low number of live cells when assessed by flow cytometry (related to step 7).

Potential solution

If cells become too confluent, there can be increased cell death. Collect iAT1s for flow cytometry or other analysis within 16 days of the medium change to L + DCI (19 days after passaging). Additionally, vigorous pipetting during dissociation leads to increased cell death. Allow most of the digestion to be enzymatic, such that pipetting only 3-5 times results in a single cell solution.

Problem 5

Difficulty in assessing maturation of generated iAT1s (related to step 2c).

Potential solution

Gene expression of AT1 marker genes in generated iAT1s can be benchmarked against both iAT2s as well as RNA extracts from primary control adult human lung tissue (see below) to assess maturation. The AT1 marker genes *AGER*, *CAV1*, *CLIC5*, and *PDPN* can be used to compare iAT1s to controls using reverse transcription quantitative PCR (RT-qPCR). Relative gene expression should be normalized to an 18S control for each sample, and fold change of iAT1 gene expression over control cells can then be calculated by $2^{-\Delta\Delta Ct}$. We recommend assigning a fold change of 1 to iAT2s.

Because primary AT1 cells are fragile and thus challenging to reliably isolate via cell sorting, we recommend instead using RNA extracts from explanted or biopsied adult human distal lung tissue as a control rather than sorted human AT1 cells, recognizing that RT-qPCR is a bulk assay and that AT1 cells are only a small percentage of the total cell population. Thus, normalized expression values of AT1 specific genes in bulk primary controls will be lower than the expression values expected of isolated AT1 cells, but will still serve as a relevant and reliable positive control. Although AT1 marker gene expression substantially increases in the differentiation from iAT2 to iAT1, iAT1s express lower levels of *AGER* and *PDPN* transcripts than bulk primary controls (Figure 2B).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to, and will be fulfilled by, the lead contact, Darrell N. Kotton (dkotton@bu.edu).

Technical contact

Questions about the technical specifics of performing the protocol should be directed to and will be answered by the technical contact, Lauren Ayers (ljayers@bu.edu).

Materials availability

Pluripotent stem cell lines used in this study are available from the CReM iPSC Repository at Boston University and Boston Medical Center and can be found at http://www.stemcellbank.bu.edu/Catalog/Item/Home.

Data and code availability

This study did not generate datasets or codes.

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AUTHOR CONTRIBUTIONS

C.L.B. and D.N.K. developed the protocol. C.L.B., L.J.A., K.-D.A., and K.M. provided resources and performed the experiments. L.J.A., C.L.B., and D.N.K. wrote the manuscript. All authors reviewed and approved the final version.

DECLARATION OF INTERESTS

The authors have filed a patent application related to the generation of human alveolar epithelial type I cells from pluripotent stem cells detailed in this manuscript.

REFERENCES

1. Burgess, C.L., Huang, J., Bawa, P.S., Alysandratos, K.D., Minakin, K., Ayers, L.J., Morley, M.P., Babu, A., Villacorta-Martin, C., Yampolskaya, M., et al. (2024). Generation of human alveolar epithelial type I cells from pluripotent stem cells. Cell Stem Cell *31*, 657–675.e8.

2. Jacob, A., Vedaie, M., Roberts, D.A., Thomas, D.C., Villacorta-Martin, C., Alysandratos, K.D., Hawkins, F., and Kotton, D.N. (2019). Derivation of self-renewing lung alveolar epithelial type II cells from human pluripotent stem cells. Nat. Protoc. *14*, 3303–3332.

3. Gotoh, S., Ito, I., Nagasaki, T., Yamamoto, Y., Konishi, S., Korogi, Y., Matsumoto, H., Muro, S., Hirai, T., Funato, M., et al. (2014). Generation of Alveolar Epithelial Spheroids via Isolated Progenitor Cells from Human Pluripotent Stem Cells. Stem Cell Rep. *3*, 394–403.

4. Sun, Y.L., Hurley, K., Villacorta-Martin, C., Huang, J., Hinds, A., Gopalan, K., Caballero, I.S., Russo, S.J., Kitzmiller, J.A., Whitsett, J.A., et al. (2021). Heterogeneity in Human Induced Pluripotent Stem Cell–derived Alveolar Epithelial Type II Cells Revealed with ABCA3/SFTPC Reporters. Am. J. Respir. Cell Mol. Biol. *65*, 442–460.

5. Korogi, Y., Gotoh, S., Ikeo, S., Yamamoto, Y., Sone, N., Tamai, K., Konishi, S., Nagasaki, T., Matsumoto, H., Ito, I., et al. (2019). *In Vitro* Disease Modeling of Hermansky-Pudlak Syndrome Type 2 Using Human Induced Pluripotent Stem Cell-Derived Alveolar Organoids. Stem Cell Rep. *12*, 431–440.

6. Yin, B.W.T., Kiyamova, R., Chua, R., Caballero, O.L., Gout, I., Gryshkova, V., Bhaskaran, N., Souchelnytskyi, S., Hellman, U., Filonenko, V., et al. (2008). Monoclonal antibody MX35 detects the membrane transporter NaPi2b (SLC34A2) in human carcinomas. Cancer Immun. *8*, 3.

7. Hurley, K., Ding, J., Villacorta-Martin, C., Herriges, M.J., Jacob, A., Vedaie, M., Alysandratos, K.D., Sun, Y.L., Lin, C., Werder, R.B., et al. (2020). Reconstructed Single-Cell Fate Trajectories Define Lineage Plasticity Windows during Differentiation of Human PSC-Derived Distal Lung Progenitors. Cell Stem Cell *26*, 593–608.e8.

8. Alysandratos, K.-D., Russo, S.J., Petcherski, A., Taddeo, E.P., Acín-Pérez, R., Villacorta-Martin, C., Jean, J.C., Mulugeta, S., Rodriguez, L.R., Blum, B.C., et al. (2021). Patient-specific iPSCs carrying an SFTPC mutation reveal the intrinsic alveolar epithelial dysfunction at the inception of interstitial lung disease. Cell Rep. *36*, 109636.

9. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods *9*, 676–682.

10. Alysandratos, K.-D., Garcia-de-Alba, C., Yao, C., Pessina, P., Huang, J., Villacorta-Martin, C., Hix, O.T., Minakin, K., Burgess, C.L., Bawa, P., et al. (2023). Culture impact on the transcriptomic programs of primary and iPSC-derived human alveolar type 2 cells. JCI Insight *8*, e158937.