Generating 3D Spheres and 2D Air-Liquid Interface Cultures of Human Induced Pluripotent Stem Cell-Derived Type 2 Alveolar Epithelial Cells

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Citation

Werder, R.B., Huang, J., Abo, K.M., Hix, O.T., Minakin, K., Alysandratos, K.D., Merritt, C., Berthiaume, K., Alber, A.B., Burgess, C.L., Kotton, D.N., Wilson, A.A. Generating 3D Spheres and 2D Air-Liquid Interface Cultures of Human Induced Pluripotent Stem Cell-Derived Type 2 Alveolar Epithelial Cells. *J. Vis. Exp.* (182), e63875, doi:10.3791/63875 (2022).

Date Published

April 15, 2022

DOI

10.3791/63875

URL

jove.com/video/63875

Abstract

In the lung, the alveolar epithelium is a physical barrier from environmental stimuli and plays an essential role in homeostasis and disease. Type 2 alveolar epithelial cells (AT2s) are the facultative progenitors of the distal lung epithelium. Dysfunction and injury of AT2s can result from and contribute to various lung diseases. Improved understanding of AT2 biology is, thus, critical for understanding lung biology and disease; however, primary human AT2s are generally difficult to isolate and limited in supply. To overcome these limitations, human induced pluripotent stem cell (iPSC)-derived type 2 alveolar epithelial cells (iAT2s) can be generated through a directed differentiation protocol that recapitulates *in vivo* lung development. iAT2s grow in feeder-free conditions, share a transcriptomic program with human adult primary AT2s, and execute key functions of AT2s such as production, packaging, and secretion of surfactant. This protocol details the methods for maintaining self-renewing iAT2s through serial passaging in three-dimensional (3D) culture or adapting iAT2s to air-liquid interface (ALI) culture. A single-cell suspension of iAT2s is generated before plating in 3D solubilized basement membrane matrix (hereafter referred to as "matrix"), where they self-assemble into monolayered epithelial spheres. iAT2s in 3D culture can be serially dissociated into single-cell suspensions to be passaged or plated in 2D ALI culture. In ALI culture, iAT2s form a polarized monolayer with the apical surface exposed to air, making this platform readily amenable to environmental exposures. Hence, this protocol generates an inexhaustible supply of iAT2s, producing upwards of 1 x 10^{30} cells per input cell over 15 passages while maintaining the AT2 program indicated by $SFTPC^{tdTomato}$ expression. The resulting cells represent

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a reproducible and relevant platform that can be applied to study genetic mutations, model environmental exposures, or screen drugs.

Introduction

In the lung, the airway and alveolar epithelial cells are the first to encounter inhaled environmental exposures, including pathogens transmitted via inhaled aerosols and noxious stimuli such as cigarette smoke. Type 2 alveolar epithelial cells (AT2s) are essential in maintaining lung homeostasis as they are the facultative progenitors of the distal lung epithelium, produce surfactants to relieve alveolar surface tension, and mount the innate immune response to inhaled exposures[1,2]. However, AT2 dysfunction can result from lung injuries or mutations in genes that are selectively expressed in AT2s, such as *SFTPC*, *SFTPB*, and *ABCA3*[3,4,5]. Previous approaches for studying these genetic mutations have relied on mouse models[6] or engineered, mutation-containing vectors introduced into immortalized cell lines[7]. Therefore, platforms that can model the effects of genetic and environmental perturbations on AT2s in the physiologically relevant cell types and in a productive *in vitro* system are needed to understand further the role AT2s play in health and disease.

In terms of modeling airborne exposures, air-liquid interface (ALI) cultures of primary airway epithelial cells have been successfully utilized to reveal key molecular responses to cigarette smoke[8] and to model airway infection[9,10]. A comparable ALI culture system for the alveolar epithelium, a key site of infection or injury in disease, is much less developed compared to the airway epithelial model system. Immortalized cell lines have been cultured at ALI as a proxy for the alveolar epithelium[11,12], but these cell lines are transcriptomically distinct from primary alveolar epithelial cells[13] and lack key cellular machinery, such as the ability to secrete surfactants or form tight junctions at ALI[12]. Primary human AT2s can be cultured in 3D spheres in the presence of fibroblasts[1,14] but are subject to limitations, including the limited accessibility from explant lungs and their tendency to senesce or lose cellular phenotype in most cultures to date. These characteristics present barriers to the widespread adoption of primary AT2 *in vitro* studies, although recent progress has been made in optimizing feeder-free 3D cultures for the expansion of primary AT2s[15,16,17,18].

Directed differentiation protocols have been developed to recapitulate *in vivo* developmental milestones to generate human induced pluripotent stem cell (iPSC)-derived type 2 alveolar epithelial cells (iAT2s)[19]. iAT2s grow as self-renewing spheres in 3D serum-free culture in a defined medium containing CHIR99021, KGF, dexamethasone, cAMP, and 3-isobutyl-1-methylxanthine (IBMX), termed "CK + DCI"[19], in the absence of fibroblast feeders and can be cultured for >20 passages[20,21]. Moreover, iAT2s share a transcriptomic program with human adult primary AT2s, form lamellar bodies, and produce and package surfactant[19,21,22]. This protocol details the serial passaging of iAT2s by dissociating the cells to a single-cell suspension. At this point, iAT2s can be replated and expanded further in 3D culture or plated in 2D ALI culture[23]. These methods can be used to study the intrinsic biology of AT2s in homeostasis and disease[20,22] and to interrogate the effects of compounds

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or stimuli in a scalable, physiologically relevant platform[23,24], as has previously been shown.

Protocol

All experiments involving the differentiation of human iPSC lines were performed in compliance with the Institutional Review Board of Boston University (protocol H33122). The dermal fibroblasts, procured for reprogramming to iPSCs, were obtained from a donor with written informed consent, under the approval of the Human Research Protection Office of Washington University School of Medicine, St. Louis, MO. Reprogrammed iPSCs were generated at the Center for Regenerative Medicine at Boston University and Boston Medical Center, Boston, MA.

1. Alveolosphere dissociation

1. Prepare complete serum-free differentiation media (cSFDM) as per the composition mentioned in **Table 1**.
2. Prepare CK + DCI media in the prepared cSFDM base as per **Table 2**.
3. Thaw 2D (human embryonic stem cell-qualified) and/or 3D (growth-factor reduced) matrix on ice as required for the experimental needs.
4. Aspirate all the CK + DCI medium using a pipette or aspirating pipette with vacuum from the 3D matrix droplets containing alveolospheres, derived from directed differentiation[19], in a 12-well plate.
5. Add 1 mL of dispase (2 mg/mL) per droplet. Gently pipette the droplet into the dispase using a P1000 pipette. Incubate at 37 °C for 1 h, pipetting up and down once after 30 min.
6. Transfer the dissociated organoids (from Step 1.5) from one matrix droplet in the dispase to a 15 mL conical tube. To wash, add 10 mL of Iscove's Modified Dulbecco's Medium (IMDM, see **Table of Materials**).
7. Centrifuge at 300 x *g* for 5 min at room temperature. Aspirate the supernatant using a pipette or aspirating pipette with vacuum, leaving as little supernatant as possible.
   NOTE: It is important to remove all dispase as any remaining dispase may dissolve the matrix that the cells will subsequently be seeded into. If a clear haze is seen above the pellet, the dispase has not completely dissolved the matrix, and more dispase can be added to the pellet for another 20-30 min at 37 °C.
8. Resuspend the cells in 1 mL of 0.05% trypsin per droplet and transfer back to the 12-well plate. Incubate at 37 °C for 12-15 min. Observe the dissociation under a microscope. Avoid over-pipetting the cells at this stage.
   NOTE: At the end of incubation, the cells need to achieve a single-cell suspension after pipetting 3-5 times with a P1000 pipette. For passaging iAT2s to ALI (Step 3), the trypsinization time needs to be minimized (maximum 12 min), such that the cells are in 2-3-cell clumps rather than single-cell suspension when ready for plating onto the cell culture insert.
9. Stop the action of trypsin with an equal volume of FBS-containing medium (10% ES-qualified FBS in DMEM). Centrifuge at 300 x *g* for 5 min at room temperature.
10. Wash the cells with 10 mL of IMDM. Centrifuge at 300 x *g* for 5 min at room temperature.
11. Resuspend the cells in an appropriate volume for counting, and then count the cells using a hemocytometer (see **Table of Materials**).

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NOTE: From one confluent 50 µL matrix droplet seeded at 400 cells/µL, the expected yield is 500,000 to 1.5 x 10^6 cells per droplet.

12. Use the single-cell suspension of iAT2 cells to generate alveolospheres by plating in the 3D matrix (Step 2) and/ or plating on cell culture inserts for ALI culture (Step 3).

2. 3D plating of iAT2s

1. After counting (Step 1.11), determine the number of desired cells to replate in the 3D matrix (400 cells/µL of the matrix with 50-100 µL of 3D matrix droplets per well of a 12-well plate). Centrifuge the cells at 300 x *g* for 5 min at room temperature. Remove as much supernatant as possible using a pipette.
2. Resuspend the cells in the 3D matrix. Resuspend quickly and on ice, if needed, to prevent the matrix from polymerizing (which occurs when warm).
3. Use a P200 pipette to dispense one 3D matrix droplet per well into a pre-warmed 12-well plate. Pipette carefully to avoid creating bubbles in the matrix droplet. Do not allow the cell suspension to settle while dispensing multiple droplets.
4. Place the plate in a 37 °C incubator for 20-30 min to allow the matrix droplets to polymerize.
5. Add 1 mL of CK + DCI + 10 µM of Y-27632 medium (see **Table of Materials**) per well to cover the matrix droplet.
6. After 72 h, change the medium to CK + DCI without 10 µM of Y-27632.
7. Replace the medium with fresh CK + DCI every 48-72 h.
NOTE: iAT2s will typically need to be passaged approximately every 10-14 days, depending on cell line and plating density.

3. Passaging of iAT2s to ALI

1. Prepare freshly coated 6.5 mm cell culture inserts 1 h before use by diluting the 2D matrix in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/ F12) to working solution as per manufacturer's instructions for the 2D matrix (see **Table of Materials**). Then, add 100 µL of the diluted matrix per 6.5 mm cell culture insert. Allow the matrix to polymerize in a 37 °C incubator for 30 min or at room temperature for 1 h.
2. Aspirate the excess matrix from the cell culture inserts using a pipette or aspirating pipette with vacuum and rinse once with DMEM/F12. Aspirate this wash immediately before adding the cells.
3. After counting, determine the number of required cells to seed the desired number of cells in the culture inserts (520,000 live cells/cm^2, equivalent to 172,000 live cells per 6.5 mm cell culture insert). Centrifuge the cells at 300 x *g* for 5 min at room temperature. Remove as much supernatant as possible.
4. Resuspend the cells in the appropriate volume to seed with 100 µL of CK + DCI + 10 µM Y-27632 per cell culture insert (cell suspension should be 1,720 cells/ µL). Add 100 µL to the apical compartment of the cell culture insert. Gently agitate the plate in a cross-pattern to ensure an even distribution of cells across the cell culture insert, and confirm this by checking under a microscope at 4x objective.
NOTE: Seeding density is critical and may require optimization, ranging from 160,000-300,000 cells per 6.5 mm cell culture insert. Calcein green (see **Table of Materials**) cell-permeable dye may be added to the cells

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and viewed on an inverted fluorescent microscope to visualize the cells after seeding.

5. Add 500 µL of CK + DCI + 10 µM of Y-27632 to the basolateral compartment of each cell culture insert.
6. Aspirate apical CK + DCI + 10 µM of Y-27632 medium 48 h after seeding using a pipette to initiate ALI (known as "air-lift").
   NOTE: The cells should be 100% confluent at this point. If the cells are not 100% confluent, Steps 3.7-3.8 should still be performed at the indicated time points; confluent cell monolayers may take longer to form.
7. Change the basolateral CK + DCI + 10 µM of Y-27632 medium after 72 h to CK + DCI without 10 µM Y-27632.
   NOTE: There should be minimal if any, "leak" of medium to the apical side. However, if this does occur in the first few days, continue to aspirate the apical side daily until there is no further leakage.
8. Replace the basolateral medium with fresh CK + DCI every 48-72 h.
   NOTE: The iAT2s used in ALI cultures are maximally matured at 5-14 days post-plating.
9. Maintain the cells with careful monitoring for up to 28 days post-plating, if needed, for more prolonged experimentation.
   NOTE: Visible signs of ALI failure, such as peeling of the cells away from the edge of the insert or formation of holes in the monolayer, may be observed after an overextended time in culture, at which point the ALI is no longer usable for experiments.

Representative Results

The iAT2s were passaged to a single-cell suspension and then replated in 3D matrix or in 2D on cell culture inserts for ALI culture (**Figure 1**). After single-cell dissociation, the iAT2s were analyzed by flow cytometry. Briefly, the cells resuspended in 1% fetal bovine serum (FBS) in phosphate-buffered saline (PBS) with calcein blue viability dye (1:1000) were analyzed on a flow cytometer, gating for non-fragments, singlets, and tdTomato. Here, as an example, is the SPC2 line (SPC2-ST-B2 clone[20]), which has a tdTomato reporter targeted to the endogenous *SFTPC* locus for ease of visualization and tracking of the AT2 program over time in culture (**Figure 2A**). The iAT2 SFTPC-tdTomato expression was maintained when replated in the 3D matrix as spheres (**Figure 2B**) and when plated on cell culture inserts (**Figure 2C**). Trans-epithelial electrical resistance (TEER) can be measured to determine the integrity of the ALI culture (**Figure 2D**). To measure TEER, a voltohmmeter was used (see **Table of Materials**), with 100 µL of CK + DCI medium added to the apical chamber. The cell culture inserts coated with Matrigel in the absence of seeded cells were treated as blanks. Readings were taken at three locations in every well.

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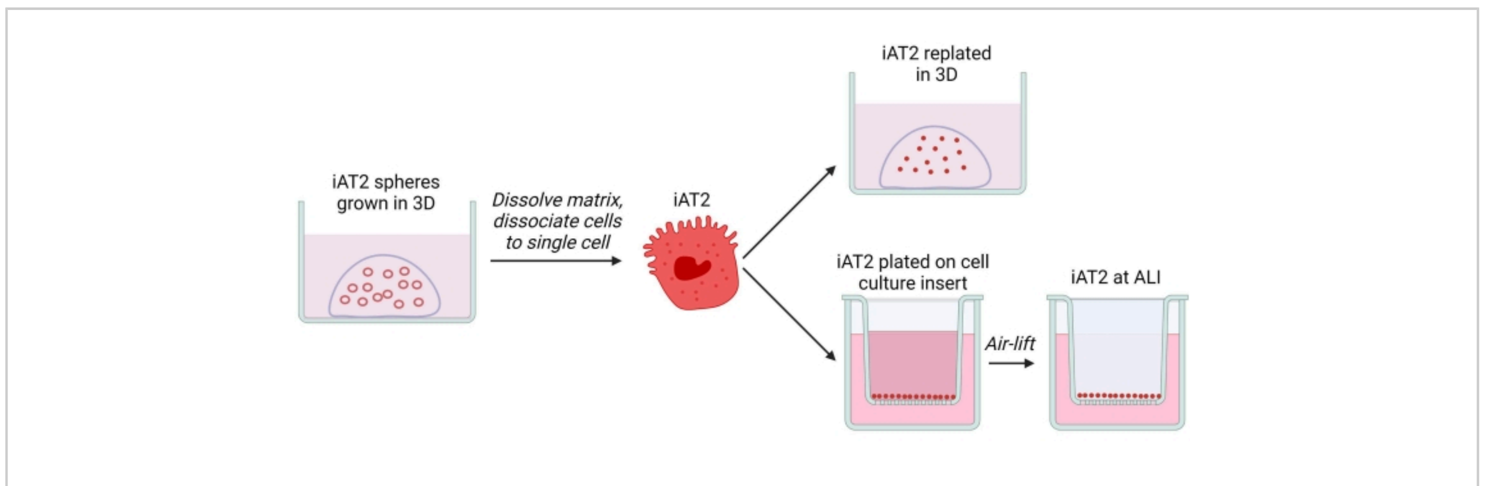

Figure 1: Schematic representation of the protocol workflow. Please click here to view a larger version of this figure.

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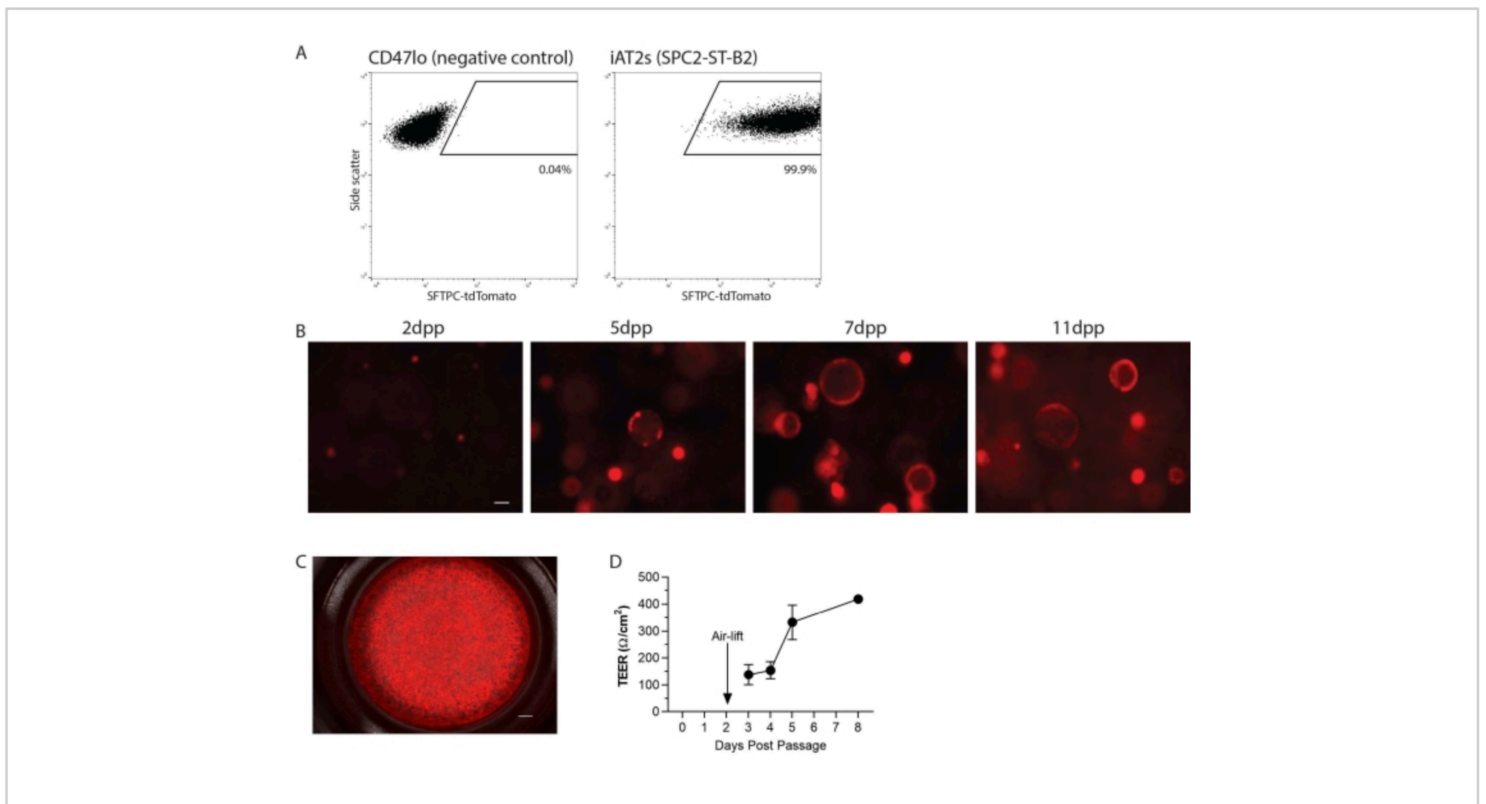

Figure 2: Representative results of the protocol. (**A**) Representative flow cytometry results for SFTPC-tdTomato in human induced pluripotent stem cell-derived type 2 alveolar epithelial-like cells (iAT2s) cultured in 3D. The negative control shown is non-lung endoderm (CD47lo). (**B**) Representative live-cell imaging of iAT2s cultured in 3D at various days post passage (dpp) (SFTPC-tdTomato, scale bar = 50 µm). (**C**) Representative live-cell imaging of iAT2s plated at air-liquid interface (SFTPC-tdTomato, scale bar = 500 nm). (**D**) Representative trans-epithelial electrical resistance of iAT2s plated at the air-liquid interface. n = 3, error bars indicate standard deviation. Please click here to view a larger version of this figure.

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Reagents	Volume for 500 mL	Final concentration
Iscove's Modified Dulbecco's Medium (IMDM)	375 mL	75%
Ham's F-12 Nutrient Mixture (F12)	125 mL	25%
B-27 (with RA) supplement	5 mL	1%
N-2 supplement	2.5 mL	0.50%
BSA (7.5% stock)	3.3 mL	0.05%
Primocin (50 mg/mL stock)	1 mL	100 µg/mL
Glutamax (100x stock)	5 mL	1x
Ascorbic Acid (50 mg/mL stock)	500 µL	50 µg/mL
1-Thioglycerol (MTG) (from 26 µL in 2 mL IMDM)	1.5 mL	4.5×10^{-4} M

Table 1: Composition of the complete serum-free differentiation media (cSFDM).

Reagent	Final concentration
CHIR99021	3 µM
rhKGF	10 ng/mL
Dexamethasone	50 nM
8-bromoadenosine 30,50-cyclic monophosphate sodium salt (cAMP)	0.1 mM
3-Isobutyl-1-methylxanthine (IBMX)	0.1 mM

Table 2: Compositions of the CK + DCI media.

Discussion

AT2s maintain lung homeostasis, and dysfunction of these key alveolar cells can both cause and result from various lung diseases. Due to the difficulty of accessing and isolating primary human AT2s, iAT2s are generated. By applying the directed differentiation methods described elsewhere[25] and the expansion and cell seeding described here, iPSCs generated from any individual can be differentiated into robustly self-renewing iAT2s, thus providing patient-specific cells for biomedical research, including basic biomedical research developmental studies, disease modeling, cell-based therapies, or drug screens. The present protocol

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details a method for dissociating iAT2s to a single-cell suspension, which can then be replated in 3D Matrigel to generate alveolospheres for cell expansion or replated in 2D ALI culture for further experiments.

The protocol has several critical steps to ensure successful passaging and replating in both 3D and ALI cultures. Mechanical trituration must be minimized, as excessive pipetting can decrease cell viability. In addition, the seeding density of iAT2s in both 3D and 2D ALI cultures is critical; for 3D culture, in general, 400 cells/µL of 3D Matrigel is optimal for most iPSC lines. However, a range of densities from 100-500 cells/µL has, at times, been successful, and optimizing the density may be required for different cell lines. For ALI culture, optimizing the seeding density of iAT2s on the cell culture inserts is also essential to achieve a confluent monolayer of cells 48 h after seeding (i.e., on the day of air-lift). Some iAT2 lines require more cells per insert; thus, if troubleshooting is required, a range from 160,000-300,000 cells/insert for 24-well cell culture inserts is recommended. 3D cultures can also be scaled to 24- and 48-well plates by maintaining the seeding density at 400 cells/µL of 3D matrix and reducing matrix droplet size to 25 µL and 20 µL, respectively. ALI cultures can be scaled to 96-well cell culture inserts by coating each insert with 30 µL of the matrix, plating 80,000 cells in 30 µL per insert, and feeding with 150 µL of basolateral media per well. Seeding density and matrix and media volumes need to be scaled and optimized accordingly for other plate formats.

A limitation of this protocol is that the cells used for ALI plating have to be sufficiently purified to be NKX2-1+ and SFTPC +[19,20,21,23] to form iAT2 ALIs, and ALI culture formation may vary from line to line. In addition, this protocol allows expansion and generation of AT2-only cultures, providing a reductionist model system based on a single key alveolar cell type. Importantly, other relevant alveolar cell types, such as alveolar type 1 cells, were missing from these platforms. Other groups have had success culturing primary human AT2s as spheroids or organotypic cultures with or without fibroblasts[1,14,15,16,17]; however, primary human AT2s tend to lose their expression of key surfactants when cultured in normal 2D culture without ALI conditions[26]. Furthermore, recent work has shown that iAT2s express higher proliferative markers and lower AT2 maturation markers than fresh, uncultured human primary AT2s[27]. Despite these differences between iAT2s and fresh primary human AT2s, we found that even cultured primary AT2s displayed transcriptomic differences from fresh, uncultured primary AT2s[27], and thus conclude that these *in vitro* models have various strengths and limitations for modeling *in vivo* lung biology.

The iAT2 platform can be applied to study genetic mutations from patient-derived iPSCs[19,20]. The system could also be substantially scaled up to accommodate high throughput drug screening. In addition, iAT2 ALIs are suitable for environmental exposures such as viral or bacterial infection or exposure to cigarette smoke, e-cigarette vapor, or other aerosols[23]. In summary, this protocol for generating both 3D and 2D ALI cultures of iAT2s allows for long-term culture of a disease-relevant cell type and provides a physiological, scalable platform that enables the usage of these cells in many applications.

Disclosures

The authors have nothing to disclose.

Acknowledgments

We sincerely thank members of the Wilson, Kotton, and Hawkins laboratories for their helpful discussions. We

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are also very grateful to Greg Miller (CReM Laboratory Manager) and Marianne James (iPSC Core Manager) for their invaluable support. We thank Brian Tilton and the BUMC Flow Cytometry Core for their technical assistance with cell sorting (supported by NIH grant #1S10OD021587-01A1). This work was supported by a CJ Martin Early Career Fellowship from the Australian National Health and Medical Research Council awarded to RBW; NIH grant F30HL147426 to KMA; an I.M. Rosenzweig Junior Investigator Award from The Pulmonary Fibrosis Foundation and an Integrated Pilot Grant Award through Boston University Clinical & Translational Science Institute (1UL1TR001430) to KDA; Swiss National Science Foundation (P2ELP3_191217) to ABA; NIH grants U01HL148692, U01HL134745, U01HL134766 and R01HL095993 to DNK; and NIH grants U01TR001810, R01DK101510, and R01DK117940 awarded to AAW; iPSC maintenance, banking, and sharing are supported by NIH grant NO1 75N92020C00005. Figure 1 was created using Biorender.com.

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